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BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Application Number: 10/032,717
Filing Date: October 23, 2001
Appellant(s): ABAD ET AL.

W. Murray Spruill
For Appellant

EXAMINER'S ANSWER

This is in response to the appeal brief filed 17 November 2006 appealing from the Office action mailed 18 May 2006

(1) Real Party in Interest

A statement identifying by name the real party in interest is contained in the brief.

(2) Related Appeals and Interferences

The following are the related appeals, interferences, and judicial proceedings known to the examiner that may be related to, directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal:

Application Serial Number 10/414,637, which is a divisional of the instant application.

(3) Status of Claims

The statement of the status of claims contained in the brief is correct.

(4) Status of Amendments After Final

The appellant's statement of the status of amendments after final rejection contained in the brief is correct.

(5) Summary of Claimed Subject Matter

The summary of claimed subject matter contained in the brief is correct.

(6) Grounds of Rejection to be Reviewed on Appeal

The appellant's statement of the grounds of rejection to be reviewed on appeal is correct.

(7) Claims Appendix

The copy of the appealed claims contained in the Appendix to the brief is correct.

(8) Evidence Relied Upon

Evidence Appendix A: sequence comparison between SEQ ID NOs:2 and 4.

Evidence Appendix B: Tounsi et al, 2003, J. Appl. Microbiol. 95:23-28.

Evidence Appendix C: Minshull et al

Evidence relied upon and provided by Appellant:

Lazar et al, 1988, Mol. Cell. Biol. 8:1247-1252: see Appellant's Appendix B.

Hill et al, 1998, Biochem. Biophys. Res. Comm. 244:573-577: see Appellant's Appendix C.

de Maagd et al, 1999, Appl. Environ. Microbiol. 65:4369-4374: see Appellant's Appendix D.

Angsuthanasombat et al, 2001, J. Biochem. Mol. Biol. 34:402-407: see Appellant's Appendix E.

Li et al: see Appellant's Appendix G.

(9) Grounds of Rejection

The following ground(s) of rejection are applicable to the appealed claims:

a. Claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52 and 55-64 are rejected under 35

U.S.C. 112, first paragraph, because the specification, while being enabling for nucleic acids encoding SEQ ID NO:2 and 10, expression cassettes comprising the nucleic acids, plants and seeds comprising a construct comprising the nucleic acid, and a method of using it to impact a plant pest, does not reasonably provide enablement for any nucleic acid that has 90% identity to SEQ ID NO:1, expression cassettes comprising the nucleic acid, plants and seeds comprising a construct comprising the nucleic acid and a method of using it to impact a plant pest. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims.

The claims are broadly drawn to a nucleic acid that has 90%, 93%, 94% or 95% identity to SEQ ID NO:1 and that encodes a Coleopteran pesticidal protein, expression cassettes comprising the nucleic acids, plants and seeds comprising constructs comprising the nucleic acids, and a method of using the constructs to impact a plant pest.

The instant specification, however, only provides guidance for methods of assaying the activity of *B. thuringiensis* strain 1218 and lysate against Western corn rootworm and Southern corn rootworm (examples 1 and 2); isolation of crystal protein from the strain and assaying of it for pesticidal activity against western corn rootworm (example 3); identification of two coding regions, *Cry1218-1* and *Cry1218-2* (SEQ ID NO:1 and 3, respectively, with SEQ ID NOs:27 and 28 as the genomic clones), isolated by unknown methods, as encoding proteins, SEQ ID NOs:2 and 4, respectively, that have an unspecified homology to Cry8Ba1 (example 4); production in *E. coli* of the truncated proteins, SEQ ID NOs:15 and 17, encoded by SEQ ID NOs:16 and 18, respectively, that are active against southern corn rootworm (example 4); and production of

maize-preferred coding sequences of a different truncated version of Cry1218-1 - the nucleic acid is SEQ ID NO:9, which encodes SEQ ID NO:10 (example 5). The specification also teaches making three mutant versions of truncated Cry1218-1 (SEQ ID NO:16), one of which has a truncated N-terminus (comprising amino acids 43-663 of SEQ ID NO:16), and the others in which the 4 amino acid sequence NGSR or LKMS has been inserted after amino acid 164 or replacing amino acids 164-166 - all of these mutant proteins are more effective against southern and western corn rootworm than Cry1218-1 (examples 6-7). The specification also teaches transformation of maize with SEQ ID NO:9 (examples 8 and 9).

The instant specification fails to provide guidance for how to make nucleic acids that have 90%, 93%, 94% or 95% identity to the full-length of SEQ ID NO:1 and that encode a Coleopteran pesticidal protein. The instant specification thus also fails to provide guidance for how to make expression cassettes comprising the nucleic acids, plants and seeds comprising a construct comprising the nucleic acids and a method of using it to impact a plant pest.

The instant specification fails to provide guidance for the full scope of which amino acids of SEQ ID NO:2 can be altered and to which other amino acids, and which amino acids must not be changed, to maintain Coleopteran pesticidal activity of the encoded protein. The specification also fails to provide guidance for the full scope of which amino acids can be deleted and which regions of the protein can tolerate insertions and still produce a functional enzyme.

Because nucleic acids that have 90% identity to the 3621 nucleotide long SEQ ID NO:1 would have up to 362 nucleotide substitutions, they could encode proteins with up to 362 amino acid substitutions; these proteins would have 70% identity to the 1206 amino acid long SEQ ID NO:2. The specification provides no guidance for which 362 amino acids to substitute and still

maintain Coleopteran pesticidal activity. Thus, undue trial and error experimentation would be required to make the claimed nucleic acids.

Similarly, nucleic acids that have 93% identity to SEQ ID NO:1 would have up to 253 nucleotide substitutions, nucleic acids that have 94% identity to SEQ ID NO:1 would have up to 217 nucleotide substitutions and nucleic acids that have 90% identity to SEQ ID NO:1 would have up to 181 nucleotide substitutions, producing proteins with 79%, 82% and 85% identity, respectively, to the 1206 amino acid long SEQ ID NO:2.

The specification on pg 28, lines 5-16, suggests making these nucleic acids by making conservative substitutions in the encoded protein. However, making "conservative" substitutions (*e.g.*, substituting one polar amino acid for another, or one acidic one for another) in proteins does not produce predictable results. Lazar et al (1988, Mol. Cell. Biol. 8:1247-1252) showed that the "conservative" substitution of glutamic acid for aspartic acid at position 47 reduced biological function of transforming growth factor alpha while "nonconservative" substitutions with alanine or asparagine had no effect (abstract). Similarly, Hill et al (1998, Biochem. Biophys. Res. Comm. 244:573-577) teach that when three histidines that are maintained in ADP-glucose pyrophosphorylase across several species are substituted with the "nonconservative" amino acid glutamine, there is little effect on enzyme activity, while the substitution of one of those histidines with the "conservative" amino acid arginine drastically reduced enzyme activity (see Table 1). The nucleic acids encoding all these mutated proteins, however, would have much greater than 90% identity to the nucleic acids encoding the original protein.

Making amino acid substitutions in *cry* proteins is also unpredictable. Each *cry* protein only has activity against one or few insect species (de Maagd et al, 1999, Appl. Environ.

Microbiol. 65:4369-4374, see pg 4369, column 1, paragraph 1), and even conservative substitutions in nonconserved regions can have unexpected effects on protein function (Figs 2 and 3). Even a single amino acid substitution in a *cry* protein may alter its insecticidal specificity, and toxicity must be determined empirically (Tounsi et al, 2003, J. Appl. Microbiol. 95:23-28; see pg 27, column 2, paragraph 2). For example, a conservative substitution of a lysine for an arginine in a cry11A protein eliminated toxicity to *Aedes aegyptii* but not other pests to which the wild-type protein has toxicity (Angsuthanasombat et al, 2001, J. Biochem. Mol. Biol. 34:402-407, paragraph spanning the columns on pg 405).

The specification, on pg 65, lines 12-14, indicates that the instant SEQ ID NO:1 has homology to GenBank U04365, which is identical to SEQ ID NO:3 of Michaels et al (1996, US Patent 5,554,534). This nucleotide sequence has 85.1% identity to SEQ ID NO:1. The specification, on pg 29, suggests using GenBank U04365 (SEQ ID NO:3 of Michaels et al) as the other nucleic acid in shuffling to create the claimed nucleic acids; however, that nucleic acid encodes a protein with 79.8% identity to the instant SEQ ID NO:2 (see sequence search report). Thus, this sequence cannot be used as guidance for nucleic acids with 90% identity to SEQ ID NO:1 and that encode proteins with 70% identity to SEQ ID NO:2, as encompassed by the full scope of the claims.

Furthermore, SEQ ID NO:3 of Michaels et al is pesticidal for *Cyclocephala borealis* (northern masked chafer) and *Popillia japonica* (Japanese beetle)(column 14, line 46, to column 15, line 2), whereas the instant SEQ ID NO:1 is pesticidal to *Diabrotica longicornis howardi* (southern corn rootworm). Given the teachings of Tounsi et al, de Maagd et al, and Angsuthanasombat et al, one of skill in the art could not even predict for which pests the protein

encoded by nucleic acids that have 90% identity to SEQ ID NO:1 would be toxic, and the specification does not teach which amino acid substitutions must can be made to retain southern corn rootworm pesticidal activity.

Given the claim breath, unpredictability, and lack of guidance as discussed above, undue experimentation would have been required by one skilled in the art to develop and evaluate nucleic acids with 90% identity to SEQ ID NO:1. a trail and error approach to making the claimed nucleic acids may require one to make all possible single amino acid substitutions in SEQ ID NO:1. Making all possible single amino acid substitutions in an 3621 nucleotide long nucleic acid like that of SEQ ID NO:1 would require making and analyzing 19^{3621} nucleic acids (or 2.3×10^{4630} nucleic acids; for comparison, the estimate of the total number of atoms in the universe is 10^{81}). These nucleic acids would have about 99.99% identity to SEQ ID NO:1. Because nucleic acids that have 90% identity to SEQ ID NO:1 would have up to 362 nucleotide substitutions, many more than 19^{3621} nucleic acids would need to be made and analyzed without further guidance.

Guo et al (2004, Proc. Natl. Acad. Sci. USA 101: 9205-9210) teach that while proteins are fairly tolerant to mutations resulting in single amino acid changes, increasing the number of substitutions additively increases the probability that the protein will be inactivated (pg 9209, right column, paragraph 2). Thus, making and analyzing proteins with up to 362 amino acid substitutions that also have pesticidal activity would require undue experimentation.

As the specification does not describe the transformation of any plant with nucleic acids with 90% identity to SEQ ID NO:1 and encoding Coleopteran pesticidal proteins within the full scope of the claims, undue trial and error experimentation would be required to screen through

the myriad of nucleic acids encompassed by the claims and plants transformed therewith, to identify those that could control plant pests, if such plants are even obtainable.

Given the claim breath, unpredictability in the art, undue experimentation, and lack of guidance in the specification as discussed above, the instant invention is not enabled throughout the full scope of the claims.

b. Claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52 and 55-64 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

A full review of the specification indicates that nucleic acids that have 90%, 93%, 94% or 95% identity to SEQ ID NO:1 and that encode Coleopteran-pesticidal proteins are essential to the operation of the claimed invention.

The level of skill and knowledge in the art at the time of filing was such that while large number of Coleopteran pesticidal proteins were known, nucleic acids that have 90%, 93%, 94% or 95% identity to SEQ ID NO:1 and that encode such Coleopteran-pesticides were not.

The claim is directed to a genus of nucleic acids that have 90%, 93%, 94% or 95% identity to SEQ ID NO:1. Nucleic acids that have 90%, 93%, 94% or 95% identity to SEQ ID NO:1 would have up to 362 nucleotide substitutions, they could encode proteins with up to 362, 253, 217 or 181 amino acid substitutions relative to the to the 1206 amino acid long SEQ ID NO:2.

The structural features that distinguish Coleopteran-pesticide-encoding nucleic acids that have 90%, 93%, 94% or 95% identity to SEQ ID NO:1 from other that have 90%, 93%, 94% or

95% identity to SEQ ID NO:1 are not described in the specification. The specification recites no structure required for Coleopteran-pesticide activity. The necessary and sufficient structural elements of a protein with Coleopteran-pesticidal activity are not described.

The only species described in the specification is SEQ ID NO:1, which encodes SEQ ID NO:2, a truncated version of SEQ ID NO:1, and a few nucleic acids with a few nucleotide substitutions relative to SEQ ID NO:1.

One of skill in the art would not recognize that Applicant was in possession of the necessary common attributes or features of the genus in view of the disclosed species. Since the disclosure fails to describe the common attributes that identify members of the genus, and because the genus is highly variant, SEQ ID NO:1 alone is insufficient to describe the claimed genus.

Hence, Applicant has not, in fact, described nucleic acids with 90%, 93%, 94% or 95% identity to SEQ ID NO:1 and that encode a protein pesticidal for at least one pest belonging to the order Coleoptera within the full scope of the claims, and the specification fails to provide an adequate written description of the claimed invention.

Therefore, given the lack of written description in the specification with regard to the structural and physical characteristics of the claimed compositions, it is not clear that Applicant was in possession of the genus claimed at the time this application was filed.

(10) Response to Argument

a. Claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52 and 55-64 do not meet the full enablement requirement of 35 U.S.C. 112, first paragraph.

The claimed invention is not fully taught in the specification.

Appellant argues that sequences that fall within the scope of the claims, guidance for making alterations, methods for assaying, a discussion of Cry-8-like endotoxins, guidance for determining percent identity and specific mutations that fall within the scope of the claims (Brief, pg 6-7).

However, the guidance is insufficient, given the scope of the claims.

Limiting the percent identity of the claimed nucleic acid and requiring a function do not teach which amino acid substitutions may be made in the proteins. The guidance on pg 17-18 and 33-37 merely discusses fragment size, percent identity, and calculation of percent identity. However, guidance for determining percent identity does not teach which amino acid substitutions are permissible. The guidance fails to sufficiently teach which 362 amino acid substitutions to make in SEQ ID NO:2, given the unpredictability in making amino acid substitutions in *Cry* proteins.

Appellant argues that they teach multiple truncated variants of SEQ ID NO:1, as shown in the Brief's Table, and the variants have 38-92% identity over the full-length of SEQ ID NO:1, as calculated on a program available over a web-site; a copy of that webpage Is not in the case (Brief, pg 7-8).

However, the specification does not teach nucleic acid within the full scope of the claims.

First, SEQ ID NO:27 is the genomic sequence. It encodes SEQ ID NO:2, and, as indicated by the rejection, nucleic acids encoding SEQ ID NO:2 are enabled. SEQ ID NO:9 is maize-optimized sequence encoding the truncation, SEQ ID NO:10. Although not claimed, nucleic acids encoding SEQ ID NO:10 are enabled.

Second, it is worth noting that the nucleic acids themselves are not pesticidal for Coleopterans; the proteins they encode are. These proteins are summarized in Table A below:

TABLE A.

Protein	Corresponds to	Same sequence as	Encoded by
SEQ ID NO:2	full-length Cry1218-1 protein		SEQ ID NO:1, SEQ ID NO:27
SEQ ID NO:6	amino acids 1-667 of SEQ ID NO:2		SEQ ID NO:5
SEQ ID NO:16	amino acids 1-669 of SEQ ID NO:2	SEQ ID NO:10	SEQ ID NO:15. SEQ ID NO:9
SEQ ID NO:12	amino acids 1-669 of SEQ ID NO:2 with NGSR inserted between amino acids 164 and 165		SEQ ID NO:11
SEQ ID NO:22	amino acids 1-669 of SEQ ID NO:2 with LKMS inserted between amino acids 164 and 165		SEQ ID NO:21, SEQ ID NO:39
SEQ ID NO:24	amino acids 1-669 of SEQ ID NO:2 with amino acids 164-166 replaced with LKMS	SEQ ID NO:44	SEQ ID NO:23
SEQ ID NO:20	amino acids 48-663 of SEQ ID NO:2		SEQ ID NO:19
SEQ ID NO:30	amino acids 48-663 of SEQ ID NO:2 with NGSR inserted between amino acids 164 and 165		SEQ ID NO:29
SEQ ID NO:32	amino acids 48-663 of SEQ ID NO:2 with LKMS inserted between amino acids 164 and 165	SEQ ID NO:42	
SEQ ID NO:34	amino acids 48-663 of SEQ ID NO:2 with amino acids 164-166 replaced with LKMS		SEQ ID NO:33
SEQ ID NO:4	full-length Cry1218-2 protein		SEQ ID NO:3 SEQ ID NO:28
SEQ ID NO:8	amino acids 1-667 of SEQ ID NO:4		SEQ ID NO:7
SEQ ID NO:18	amino acids 1-673 of SEQ ID NO:4		SEQ ID NO:17

As can be seen, there is little diversity in the sequence of the proteins taught in the specification. All that is taught are SEQ ID NO:s 2 and 4, and two truncations of SEQ ID NO:2 and 4 and variants of the SEQ ID NO:2 truncations with several small mutations at one particular site in the protein. One of ordinary skill in the art knows that it is only the N-terminal half the large *Cry* proteins that is the actual toxin (see for example, de Maagd, pg 4369, left column, paragraph 1). Further, it is well-known that the full-length protein is difficult to express in plants, and expression of the truncated protein is sufficient to confer pesticidal activity to the plant, (see, for example, *Adang v. Fischhoff* 62 USPQ2d 1504). Thus, the truncated proteins are obvious from the teaching of SEQ ID NO:2.

One of skill in the art would not consider a truncated protein as having 56% identity to the full length protein, but would only look at it as a truncated protein. The local match similarity between SEQ ID NO:15 and the first half of SEQ ID NO:1 is 100%. This can be represented as in the following 20 amino acid long "protein" in which the Original amino acids are represented by "O":

Original:	OOOOOOOOOOOOOOOOOOOO
Truncated with 50% query match similarity	OOOOOOOOOO

One of skill in the art would understand a protein with 50% identity to encompass the following types of proteins, where in the following "S" represents any amino acid other than the original:

Original:	OOOOOOOOOOOOOOOOOOOO
Protein with 50% identity	SOSOOSOOSOSOSOSSSOSO
Protein with 50% identity	SOOSOSOSOOSOSSOSSOOS
Protein with 50% identity	OSOSOOSOOSOSOSOSOSSO
Protein with 50% identity	OOSOOSOOSOSOSOSOSOSO

Thus, SEQ ID NO:16 does not teach a protein with 56% identity to SEQ ID NO:2, and SEQ ID NO:15, the nucleic acid that encodes SEQ ID NO:16, does not teach a nucleic acid with 55% identity to SEQ ID NO:1.

Similarity, SEQ ID NO:20 does not teach a protein with 52% identity to SEQ ID NO:2 - it consists of amino acids 48-663 of SEQ ID NO:2 - and SEQ ID NO:21, the nucleic acid that encodes SEQ ID NO:20, does not teach a nucleic acid with 55% identity to SEQ ID NO:1. Further it's identification of SEQ ID NO:20 as having 92% local identity to amino acids 1-699 of SEQ ID No:2 is equally misleading, as SEQ ID NO:20 only spans amino acids 48-663 of SEQ ID NO:2.

That the specification provides guidance in making nucleic acids encoding proteins with truncations at amino acid 663, 699 and 667, eliminating the C-terminus, and truncations off a portion of the N-terminus (in which the first 47 amino acids are eliminated) is not disputed. However, this is not anywhere near the full scope of the genus of claimed nucleic acids.

Further, even if one were to consider SEQ ID NO:20 and 16 as teaching proteins with 52% and 56% identity to SEQ ID NO:2, they would not teach how to make proteins with 362 amino acid substitutions over the entire length of SEQ ID NO:2.

Third, the 4 amino acid long substitutions/insertions at amino acid 164 of SEQ ID NO:2 are a teaching of variants, but these variants do not teach the full scope of the claimed variants. One of skill in the art would not consider these sequences as teaching which 181, 217, 253 or 362 amino acids to substitute in SEQ ID NO:2.

Fourth, SEQ ID NO:3 differs from SEQ ID NO:1 primarily in a 400 nucleotide long region (see search results; SEQ ID NO:2 and 4 differ by only 16 amino acids over amino acids 1-514 of SEQ ID NO:2, and are identical over amino acids 655-1206 of SEQ ID NO:2). Because the vast majority of substitutions are localized to such a small area, the protein it encodes, SEQ ID NO:4, has only about 132 amino acid substitutions relative to SEQ ID NO:2. Thus, SEQ ID NO:3 cannot provide all the necessary guidance for making nucleic acids with 90% identity to SEQ ID NO:1 and encoding a protein with 362 amino acid substitutions relative to SEQ ID NO:2, as encompassed by the full scope of the claims.

In summary, the specification fails to teach how to make nucleic acids within the full scope of the claims.

Appellant argues that SEQ ID NO:19 comprises only 1860 nucleotides of the 3621 nucleotides of SEQ ID NO:1, but it still retains activity, showing that it is likely that altering one or more of the other 1761 nucleotides of SEQ ID NO:1 would not disrupt function; furthermore, SEQ ID NO:9 encodes the same protein as SEQ ID NO:19 but has maize optimized codons, and it shares 38% global identity to SEQ ID NO:1 and 68% local identity (Brief, pg 8).

However, one of skill in the art would not consider a truncated protein as providing guidance for making amino acid substitutions in SEQ ID NO:2. Substitutions in the latter half of the protein affect the folding of the entire protein, and cannot be made willy-nilly just because a protein can be truncated.

All the studies on Cry protein structure and *Cry* protein amino acid substitutions look only at the N-terminal half of the proteins (See Li et al, Tounsi et al, de Maagd et al, and Angsuthanasombat et al). There is no guidance in the art or the specification for making amino acid substitutions in the C-terminal half; because no guidance is provided, one would have to use trail and error experimentation to make nucleic acids within the full scope of the claims. Multiple amino acid substitutions within the C-terminal half of the *Cry* protein would alter overall protein structure, affecting its stability of the protein and/or availability of the protease site to insect enzymes, thus rendering the protein non-toxic.

Further, the claims encompass nucleic acids that encode Coleopteran pesticides that have substitutions over the entire length of SEQ ID NO:2, not just substitutions in one region. The specification does not teach how to make such nucleic acids.

The teachings in the specification are not sufficient for one of skill in the art to make and use the claimed invention

Appellant argues that the examiner overlooks the guidance in the specification for making nucleic acids with 90%, 93%, 94% or 95% identity to SEQ ID NO:1; guidance is provided on pg 8 and 29 and examples 4, 6 and 7 (Brief, pg 8-9).

However, the guidance provided in the specification is not sufficient to teach how to make nucleic acids within the full scope of the claims.

Pg 8 of the specification defines terms like "protein", "amino acid", isolated", and "pesticidal activity", but does not provide any guidance for making nucleic acids encoding Coleopteran pesticidal proteins with 362 amino acid substitutions relative to SEQ ID NO:2.

The specification, on pg 29, suggests using GenBank U04365 as the other nucleic acid in shuffling to create the claimed nucleic acids; however, GenBank U04365 encodes a protein with 79.8% identity to the instant SEQ ID NO:2. Thus, this sequence cannot be used as guidance for nucleic acids with 90% identity to SEQ ID NO:1 and that encode proteins with 70% identity to SEQ ID NO:2, as encompassed by the full scope of the claims.

Furthermore, SEQ ID NO:3 of Michaels et al is pesticidal for pesticidal for *Cyclocephala borealis* (northern masked chafer) and *Popillia japonica* (Japanese beetle), whereas the instant SEQ ID NO:1 is pesticidal to southern corn rootworm. Given the teachings of Tounsi et al, de Maagd et al, and Angsuthanasombat et al, one of skill in the art could not even predict for which pests the protein encoded by nucleic acids that have 90% identity to SEQ ID NO:1 would be toxic, and the specification does not teach which amino acid substitutions to make to retain proper pesticidal activity. The claims require that the protein be pesticidal for Coleopterans; the

specification does not teach the full scope of substitutions that would retain that activity.

Examples 4, 6 and 7 teach making the truncations and the two 4 amino acid substitutions presented in Table 1 of the Reply Brief. Why these examples do not teach nucleic acids within the full scope of the claims is discussed above.

Further, the specification on pg 3, lines 4-8, even acknowledges the unpredictability in making amino acid substitutions in Cry proteins:

> Although numerous investigators have attempted to make mutant endotoxin proteins with improved insecticidal activity, few have succeeded. In fact, the majority of genetically engineered B. thuringiensis toxins that have been reported in the literature report endotoxin activity that is no better than that of the wild-type protein, and in many cases, the activity is decreased or destroyed altogether.

Appellant argues that the amount of experimentation required is not undue - claim 9 requires the steps required of claim 1 plus the transformation of a plant; because plant transformation is not undue, claim 9 requires no undue experimentation. Applicant makes a similar analysis for claim 17 (Brief, pg 9)

However, making nucleic acids within the full scope of the claims requires undue experimentation.

Making the nucleic acids would require undue experimentation because the specification does not provide sufficient guidance as to which 362 amino acid substitutions can be made in SEQ ID NO:2. Thus, one would need to randomly make nucleic acids encoding proteins with 362 amino acid substitutions and test them. Because the lack of guidance in the specification means this would require trail and error experimentation, because of the likelihood of protein inactivation (see Guo et al, pg 9209, right column, paragraph 2), and because of the unpredictability of amino acid interactions in *Cry* proteins (de Maagd et al, 1999, pg 4369, column 1, paragraph 1), this experimentation would be undue.

Appellant argues that SEQ ID NO:3 has 92% identity to SEQ ID NO:1, providing guidance for which residues can be mutated, and the maize-optimized SEQ ID NO:9, which has 38% global identity to SEQ ID NO:12, provides further guidance (Brief, pg 9-10).

However, SEQ ID NO:9 encodes amino acids 1-669 of SEQ ID NO:2; there are no amino acid substitutions in the encoded protein. The protein SEQ ID NO:3 encodes, SEQ ID NO:4, has only about 132 amino acid substitutions relative to SEQ ID NO:2.

Thus, none of SEQ ID NO: 3, SEQ ID NO:9 or the specification provide sufficient guidance for making nucleic acids with 90% identity to SEQ ID NO:1 and encoding a protein with 362 amino acid substitutions relative to SEQ ID NO:2.

Appellant argues that shuffling could be used to make the claimed nucleic acids, as was customary at the time of filing, citing US 5,837,458, Minshull et al and Christians et al (Brief, pg 10).

However, with respect to using DNA shuffling, the specification, on pg 29, suggests using GenBank U04365, which is identical to SEQ ID NO:3 of Michaels et al (1996, US Patent 5,554,534), as the other nucleic acid in shuffling; however, this sequence encodes a protein with 79.8% identity to the instant SEQ ID NO:2. Thus, such a nucleic acid could not be used to generate a nucleic acid that encodes a Coleopteran pesticidal protein with 70% identity to SEQ ID NO:2. Furthermore, Christians et al did not produce proteins with only 70% identity to the starting protein (see paragraph spanning pg 260-261).

Minshull et al teaches that a population should be used as the starting material (pg 284, right column, paragraph 4); the specification does not teach such a population. Minshull et al also teaches that the activities of chimeric enzymes are not predictable simply by comparing

those of the parent enzymes (paragraph spanning pg 288-289); thus, even if the population of starting materials has been provided, making nucleic acids that encode proteins pesticidal to coleopterans and have substitutions within the full scope of the claims is not predictable.

Thus, the teachings of '458, Minshull et al and Christians et al are insufficient to provide guidance for making the claimed nucleic acids, and Minshull et al argues that doing so would be unpredictable.

Appellant argues that at the time of filing it was routine to mutate amino acids in a protein then test for activity, citing Lazar et al, Hill et al, Tounsi et al, de Maagd et al, and Angsuthanasombat et al; these references show it is possible to make the claimed nucleic acids (Brief, pg 10-11).

However, although point mutations and substitutions of a few amino acids have been made in *Cry* proteins, no one has substituted 362, 253, 217 or 181 amino acids of a *Cry* protein, as encompassed the claimed nucleic acids. Angsuthanasombat, de Maagd et al, and Tounsi et al show that interactions between amino acids in *Cry* proteins is much more complex than can be predicted from guidance in the specification or the art.

de Maagd were surprised by many of their results (pg 4373, right column, paragraphs 2-3), showing that the residues have complex and unpredictable interactions with one another or with the insect receptor.

The amino acids critical to SEQ ID NO:2 activity are not taught, nor can they be predicted from the art.

Appellant argues that the specification teaches *Cry* proteins have 5 conserved blocks and 3 domains, and because the truncated protein retains activity, the deleted region would tolerate changes made using the guidance in the specification (Brief, pg 11).

However, the claims are not limited to changes made only in the deleted region, of such changes even can be made. The 5 conserved blocks and 3 domains in the N-terminal half of the protein (the non-deleted region) are not also sufficient to teach which 362 amino acid substitutions to make over the full length of the protein, as encompassed by the full scope of the claims.

Appellant argues that because assays for determining if the protein retains activity were disclosed, one could make and test the modified nucleic acids, and nothing more is required to enable the claims (Brief, pg 11-12).

However, the teachings for how to make the claimed nucleic acids are inadequate for the full scope of those nucleic acids. Thus, the making and testing cycle could be repeated ad infinitum without success.

Appellant argues that the Declaration of Dr. Abad stated that the procedures in examples 4, 6 and 7 were routine, and Lazar et al, Hill et al, Tounsi et al, de Maagd et al, and Angsuthanasombat et al support this (Brief, pg 12).

However, the procedures in examples 4, 6 and 7 only teach two truncations and two four amino acid insertions/substitution at the same site in the protein. These examples and the prior art only teach how to make a very small subset of the nucleic acids encompassed by the claims. None of these teach how to make a nucleic acid encoding a Coleopteran pesticidal protein with

362 amino acid substitutions distributed over the full length of SEQ ID NO:2, as encompassed by the claims.

The reasoning provided by the Office is well-founded.

Appellant argues that guidance is provided for which sequence alterations may be made, and the claims require both a structural component and a functional one; Appellant again refers to Table 1 of the Brief (Brief, pg 12-13).

However, Table 1 only teaches a very small subset of the claimed nucleic acids and does not provide guidance for making the full scope of the claimed nucleic acids, as discussed above. Further, a requirement that the nucleic acid has both a particular structure and a particular function does not teach which structure confers the claimed function.

Appellant argues that the analysis that one must make and test all possible combinations is incorrect, because, citing In re Borkowski, is it improper determine an appellant's claims are broader than they really are (Brief, pg 13).

However, Appellant's claims actually do encompass nucleic acids encoding Coleopteran pesticidal proteins with 362 amino acid substitutions distributed over the entire length of SEQ ID NO:2. Appellant has not denied that such nucleic acids are encompassed by their claims. Thus, *In re Borkowski* does not apply to the instant case.

Appellant argues that the specification provides guidance for making modifications, describes the domains of *Cry* proteins, provides insights into where modifications may be made, and teaches assays and variants (Brief, pg 13-14).

However, the guidance and insights are not sufficient for making nucleic acids encoding Coleopteran pesticidal proteins with 362 amino acid substitutions distributed over the entire length of SEQ ID NO:2. The variants and teaching of assays do not make up for this lack.

Appellant argues that the examiner overlooks the guidance in Li et al, as cited on pg 25 of the specification (Brief, pg 14).

However, Li et al only provides guidance for making truncations and insertion of chymotrypsin cleavage sites; Li et al do not provide guidance for making 181, 217, 253 or 362 amino acid substitutions in a 1206 amino acid protein. Further, de Maagd et al, 1999, teach that that the crystal structure of Cry1C only allows for limited prediction of the structure of the closely related Cry1Aa (pg 4373, right column, paragraph 4). Thus, Li et al would provide even less guidance for making a Cry-8 like protein, which is even less related to Cry3A than Cry1C is to Cry1Aa.

Further, the instant inventors did not use Li et al to make 181, 217, 253 or 362 amino acid substitutions in a 1206 amino acid protein to create a Coleopteran pesticidal protein.

Appellant argues that SEQ ID NO:3 is a natural variant of SEQ ID NO:1 (Brief, pg 15).

However, SEQ ID NO:3 does not encode a protein that has 362 amino acid substitutions relative to SEQ ID NO:2 - it encodes a protein that has only about 132 amino acid substitutions relative to SEQ ID NO:2. Thus, it does not provide guidance for making nucleic acids within the full scope of the claims.

Appellant argues that SEQ ID NO:19 has 52% global identity to SEQ ID NO:1, enabling claims to nucleic acids with 90% identity to SEQ ID NO:1 (Brief, pg 15).

However, SEQ ID NO:19 encodes a protein the consists of the first 669 amino acids of SEQ ID NO:2. Such truncated sequences do not provide guidance for how to make nucleic acids that encode Coleopteran pesticidal proteins with 362 amino acid substitutions relative to SEQ ID NO:2.

Appellant argues that the truncated sequences establish that a region can be deleted to produce a protein that retains activity; one of skill in the art would recognize that this region would likely tolerate modifications, as well as which regions can be deleted (Brief, pg 15-16).

However, one of skill in the art would recognize that multiple amino acid substitutions within the C-terminal half of the Cry protein would alter overall protein structure, affecting its stability of the protein and/or availability of the protease site to insect enzymes, thus rendering the protein non-toxic. Deletion is not the same thing as amino acid substitution. Furthermore, the claims are not limited to nucleic acids that encode proteins that have only amino acid substitutions in the C-terminal half of SEQ ID NO:2, but encompass nucleic acids that encode Coleopteran pesticidal proteins with 362 amino acid substitutions distributed over the entire length of SEQ ID NO:2, as well as nucleic acids that encode Coleopteran pesticidal proteins with 362 amino acid substitutions in only the N-terminal half of SEQ ID NO:2. The specification does not teach how to make any of these.

Appellant argues that the claims do not require a deletion or substitution, only that the nucleic acids have the recited percent identity (Brief, pg 16).

However, the claims encompass nucleic acids that encode Coleopteran pesticidal proteins with 362 amino acid substitutions relative to SEQ ID NO:2. They are not limited to nucleic acids that encode SEQ ID NO:2 or its truncations, SEQ ID NOs:16 and 20.

Appellant argues that the requirement that Applicant make and test every possible substitution is improper, and many changes can be made that do not change the amino acid sequence of the encoded protein (Brief, pg 16).

However, making and testing every possible substitution is not required. One must practice trial and error experimentation, however, because the guidance in the specification is so insufficient. Practicing trail and error experimentation may well mean that an impossibly large number of possible substitutions must be tested. The numbers of possible single amino acid substitutions is presented to illustrate the scale of the trail and error experimentation required.

Appellant argues that SEQ ID NO:3 provides support for substitutions that can be made (Brief, pg 16).

However, SEQ ID NO:3 does not provides support for substitutions within the full scope of the claims. SEQ ID NO:3 does not encode a protein that has 362 amino acids substitutions relative to SEQ ID NO:2 - it encodes a protein that has only about 132 amino acid substitutions relative to SEQ ID NO:2. Thus, it does not provide guidance for making nucleic acids within the full scope of the claims.

The experimentation required is undue.

Appellant argues that the examiner has not established that the experimentation required is undue and has ignored the guidance in the specification (Brief, pg 17-18).

However, the guidance in the specification is not sufficient for making nucleic acids encoding Coleopteran pesticidal proteins with 362 amino acid substitutions distributed over the

entire length of SEQ ID NO:2, as encompassed by the full scope of claims. Thus, making these nucleic acids, if it is even possible to do so, would require undue experimentation.

See *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ 2d 1016 at page 1027:

> ... despite extensive statements in the specification concerning all the analogs of the EPO gene that can be made, there is little enabling disclosure of particular analogs and how to make them. Details for preparing only a few EPO analog genes are disclosed. Amgen argues that this is sufficient to support its claims; we disagree. This "disclosure" might well justify a generic claim encompassing these and similar analogs, but it represents inadequate support for Amgen's desire to claim all EPO gene analogs. There may be many other genetic sequences that code for EPO-Type products. Amgen has told how to make and use only a few of them and is therefore not entitled to claim all of them.

The cited references support the rejection.

Appellant argues that Lazar is drawn to transforming growth factor alpha, a small mammalian peptide unrelated to *Cry* proteins and thus irrelevant to the claims; it would be unsurprising to one of skill in the art that changes in conserved amino acids would lead to loss of function and the purpose of Lazar was to study the primary structure of the protein (Brief, pg 18-19).

However, Lazar et al teach the unpredictability in making amino acid substitutions in proteins, and this unpredictability is applicable to large non-mammalian proteins. Further, Lazar et al only made single amino acid substitutions. The claims encompass nucleic acids that having 90% identity to SEQ ID NO:1 and encoding Coleopteran pesticidal proteins with 362 amino acid substitutions. Guo et al teaches that increasing the number of substitutions above single amino acid changes additively increases the probability that the protein will be inactivated (pg 9209, right column, paragraph 2). Thus, without further guidance, one of skill in the art could not make this many substitutions and still produce functional proteins.

Appellant argues that Hill is drawn to ADP-glucose pyrophosphorylase, proteins unrelated to *Cry* proteins and thus irrelevant to the claims; it would be unsurprising to one of

skill in the art that changes in conserved amino acids would lead to loss of function, and that Hill shows strictly conserved residues whose functions are essential (Brief, pg 19).

However, Hill et al teach the unpredictability in making amino acid substitutions in proteins with a number of homologs, and this unpredictability is applicable to other proteins. Hill et al made single amino acid substitutions, and found unpredictably in those; making nucleic acids that having 90% identity to SEQ ID NO:1 and encoding Coleopteran pesticidal proteins with 362 amino acid substitutions would be much more unpredictable. Guo et al teaches that increasing the number of substitutions above single amino acid changes additively increases the probability that the protein will be inactivated (pg 9209, right column, paragraph 2). Thus, without further guidance, one of skill in the art could not make this many substitutions and still produce functional proteins.

Appellant argues that de Maagd teaches that much is known about *Cry* protein structure and that essential residues have been identified in insect-specific toxicity; this knowledge can be used to make substitutions in the claimed nucleic acids (Brief, pg 20).

However, de Maagd were surprised by many of the results (pg 4373, right column, paragraphs 2-3), showing that the residues have complex and unpredictable interactions with one another or with the insect receptor. Further, de Maagd et al teach that that the crystal structure of Cry1C only allows for limited prediction of the structure of Cry1Aa (pg 4373, right column, paragraph 4). The essential residues for insect-specific toxicity must be determined for each *Cry* protein.

Appellant argues that Tounsi shows that using alignments and domain information substitutions can be made in *Cry* proteins, and Angsuthanasombat show that mutations in regions known to be essential for toxicity effect toxicity (Brief, pg 20-21).

However, Tounsi and Angsuthanasombat show that single amino acid substitutions made using alignments and domain information were unpredictable. If single amino substitutions were that unpredictable, making 362 amino acid substitutions, including in regions of the protein where no domain information is known, would be even more unpredictable.

The specification does not meet the standard for enablement.

Appellant argues that that one would only need to make the claimed variants and assay them for activity using routine methods; thus the amount of experimentation is not undue; the plant transformation steps also do not require undue experimentation (Brief, pg 22).

However, while the plant transformation steps are relatively straightforward, making nucleic acids within the full scope of the claims would require undue experimentation, given the insufficient guidance in the specification.

Appellant argues that based on the guidance of exemplary nucleic acids and proteins and methods for assaying, one of skill in the art could choose among possible sequence modifications and test them for activity; thus the quantity of experimentation is not undue, citing nonprecedential Board decisions (Brief, pg 22).

However, the number of modifications encompassed by and required by the claims would require undue experimentation in making those nucleic acids. Furthermore, Federal Circuit Rule 47.6 prohibits the citation of nonprecedential opinions.

Appellant argues that just because some experimentation is required, it does not mean that the experimentation is undue (Brief, pg 23).

However, make nucleic acids with 90%, 93%, 94% or 95% identity to SEQ ID NO:1 and encoding Coleopteran pesticidal proteins with up to 362, 253, 217 or 181 amino acid substitutions relative to SEQ ID NO:2 would require undue experimentation, given that the specification does not teach how to make such nucleic acids.

Claims 38, 43, 49, 55, 56, 58, 59, 63 and 64 are also not enabled.

Appellant argues that these claims are narrower in scope and should be considered in view of the arguments above (Brief, pg 24).

However, the specification also does not teach how to make nucleic acids with 93%, 94% or 95% identity to SEQ ID NO:1 and encoding Coleopteran pesticidal proteins with up to 253, 217 or 181 amino acid substitutions relative to SEQ ID NO:2.

b. Claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52 and 55-64 do not meet the written description requirement of 35 U.S.C. 112, first paragraph.

The claimed sequences are not adequately described in the specification.

Appellant argues that the Table in the Brief shows sequences that have been modified but still retain the activity (Brief, pg 25).

However, the sequences in the Table do not describe the structure of nucleic acid within the full scope of the claims. No nucleic acid with 90% identity to SEQ ID NO:1 and that encodes a Coleopteran pesticidal protein with 70% identity to the original protein has been described.

Appellant argues that individual support for each species is not required, only that a representative number be described (Brief, pg 25).

However, a representative number of species encompassing the full scope of the claims have not been described. The specification has not described the structure of any nucleic acid with 90% identity to SEQ ID NO:1 and that encodes a Coleopteran pesticidal protein with 362 amino acid substitutions relative to SEQ ID NO:2 , much less the full scope of such nucleic acids.

Appellant argues that the Written Description Guidelines, *Lilly* and *Enzo* state that written description requires a precise definition by structure, which is present in the 90% identity recitation (Brief, pg 26).

However, *Enzo* states: "the written description requirement would be met ... if the functional characteristics of [a genus of polypeptides] were coupled with a disclosed correlation between that function and a structure that is sufficiently known or disclosed" (emphasis added). The correlation between that function and a structure is not sufficiently known in *Cry* proteins as a whole, and the description and assays provided in the specification are insufficient.

Only a portion of the structural features have been described - the percent identity to SEQ ID NO:1. But because this includes nucleic acids in which the protein sequence has a very large number of amino acid substitutions, those amino acid substitutions that do not alter the function of the protein must be described. They are not. A representative number of nucleic acids that

have 90%, 93%, 94% or 95% identity to SEQ ID NO:1 and up to 362, 253, 217 or 181 amino acid substitutions, respectively, relative to the to the 1206 amino acid long SEQ ID NO:2 are not described.

Appellant argues that Example 14 of the Written Description Guidelines says that proteins with 95% identity to a given sequence identifier and that have a specified activity are described (Brief, pg 26-27).

However, the instant claims are not drawn to proteins with 95% identity to a given sequence identifier and that have a specified activity or to nucleic acids encoding such proteins. The claims encompass nucleic acids that encode proteins that have 70%, 79%, 82% and 85% identity to SEQ ID NO:2. Thus, the fact pattern in the instant case is different from that in Example 14 of the Written Description Guidelines.

There is insufficient written description support in the specification.

Appellant argues that the specification describes 14 variants with 38%-92% identity to SEQ ID NO:1 (Brief, pg 28-29).

However, these variants are very similar to one another. The full scope of nucleic acids encompassed by the claims are not described. For example, the specification does not describe the structure of any nucleic acid encoding a Coleopteran pesticidal protein with 362 amino acid substitutions distributed over the full length of SEQ ID NO:2.

Lilly applies to the instant case.

Appellant argues that *Invitrogen* indicates that that case is different from the fact pattern in *Lilly* and *Feirs*, and argue this would apply here (Brief, pg 29-30).

However, the fact pattern in *Invitrogen* is different from the instant case; the claims in *Invitrogen* were drawn to known proteins mutated so that one function is lost. In the instant case, the claims are drawn to proteins in which up to 30% of the amino acids are substituted but that retain the activity. The structural features of such nucleic acids are not described.

Claims 38, 43, 49, 55, 56, 58, 59, 63 and 64 are also not described.

Appellant argues that these claims are narrower in scope and should be considered in view of the arguments above (Brief, pg 30).

However, the specification also does not describe the structure of nucleic acids with 93%, 94% or 95% identity to SEQ ID NO:1 and that encode Coleopteran pesticidal proteins with up to, 253, 217 or 181 amino acid substitutions relative to SEQ ID NO:2.

(11) Related Proceeding(s) Appendix

No decision rendered by a court or the Board is identified by the examiner in the Related Appeals and Interferences section of this examiner's answer.

For the above reasons, it is believed that the rejections should be sustained.

Respectfully submitted,

Anne Kubelik

Primary Examiner

Conferees:

Anne Marie Grunberg

Supervisory Patent Examiner, Art Unit 1638

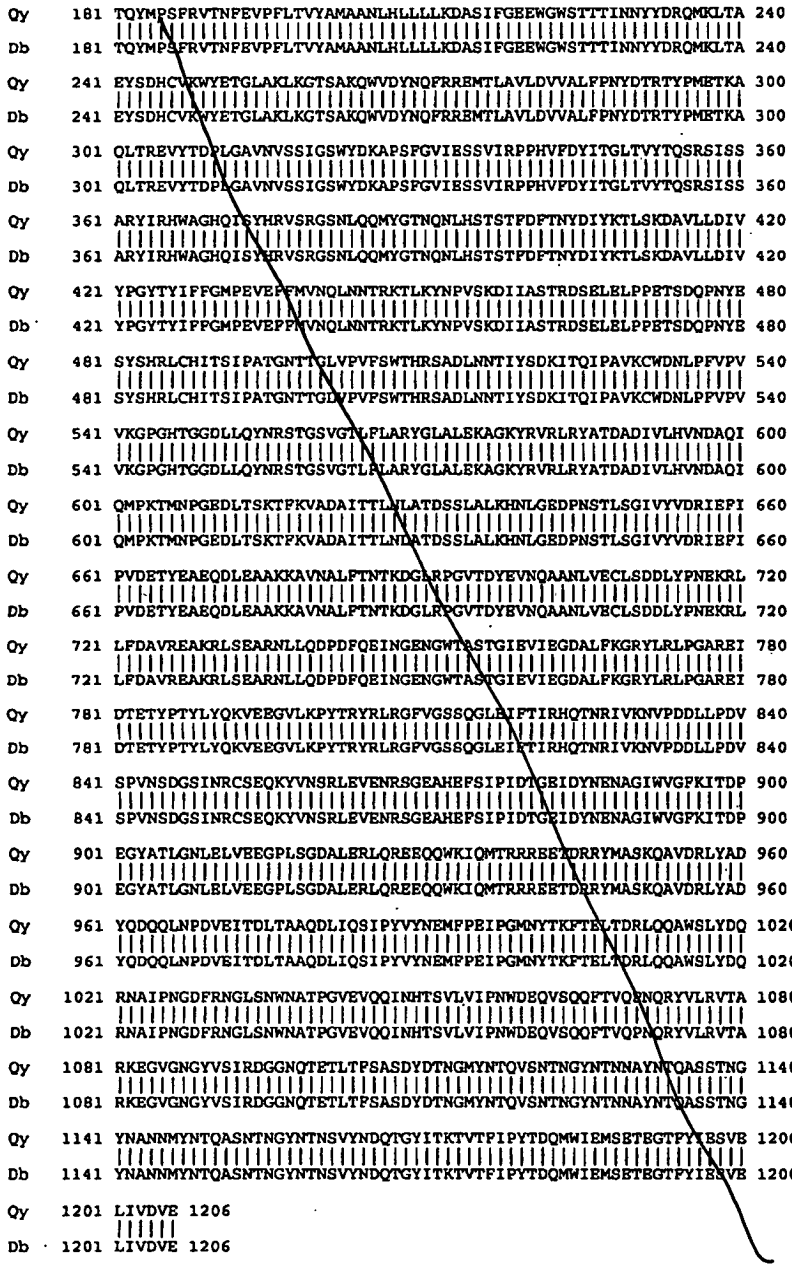
Jean Witz

Quality Assurance Specialist, TC 1600

JEAN WITZ
QUALITY ASSURANCE SPECIALIST
TECHNOLOGY CENTER 1600

Appendix A

Instant SEQ ID NO: 2 vs Instant SEQ ID NO: 4



RESULT 2
US-10-032-717-4
; Sequence 4, Application US/10032717
; Patent No. US20020151709A1
; GENERAL INFORMATION:
; APPLICANT: Andre R. Abad
; APPLICANT: Nicholas B. Duck
; APPLICANT: Xiang Feng
; APPLICANT: Ronald D. Flannagan
; APPLICANT: Theodore W. Kahn
; APPLICANT: Lynn B. Sims
; TITLE OF INVENTION: Genes Encoding No. US20020151709Alel Proteins With
; TITLE OF INVENTION: Pesticidal Activity Against Coleopterans
; FILE REFERENCE: 35718/237005
; CURRENT APPLICATION NUMBER: US/10/032,717
; CURRENT FILING DATE: 2001-10-23
; PRIOR APPLICATION NUMBER: 60/242,838
; PRIOR FILING DATE: 2000-10-24
; NUMBER OF SEQ ID NOS: 48
; SOFTWARE: FastSEQ for Windows Version 4.0
; SEQ ID NO 4
; LENGTH: 1210
; TYPE: PRT
; ORGANISM: Bacillus thuringiensis
US-10-032-717-4

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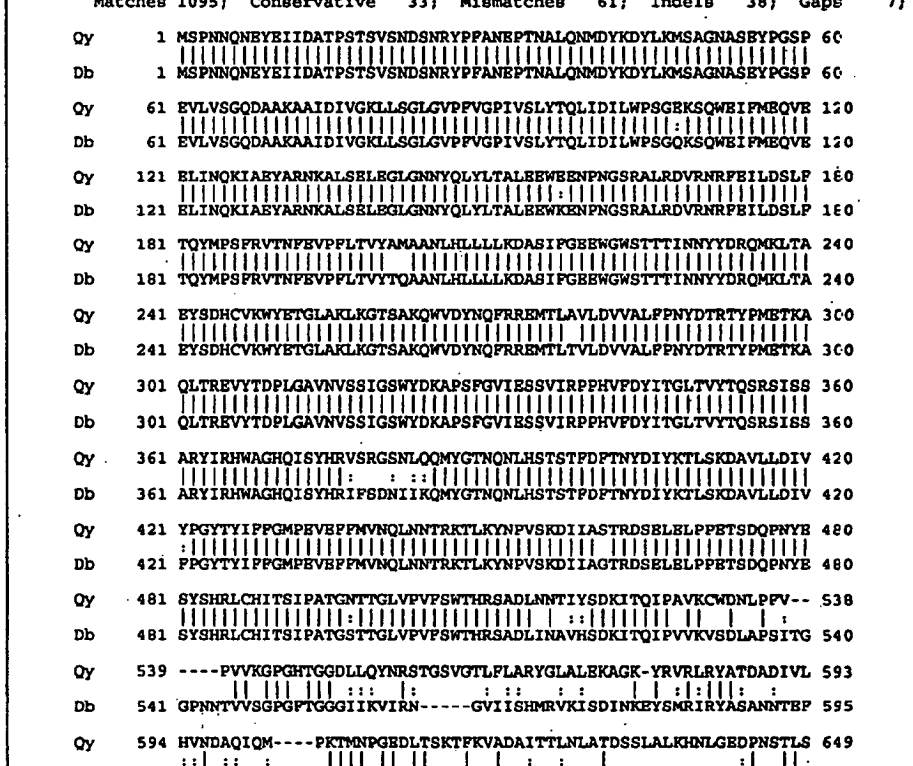

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Qy    700 NQAANLVECLSDDLYPNEKRLLFDAVREAKRLSEARNLLQDPDFQEINGENGWTASTGIE 759
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RESULT 3
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; Patent No. US20020151709A1
; GENERAL INFORMATION:
; APPLICANT: Andre R. Abad
; APPLICANT: Nicholas B. Duck
; APPLICANT: Xiang Peng
; APPLICANT: Ronald D. Flannagan
; APPLICANT: Theodore W. Kahn
; APPLICANT: Lynn B. Sims
; TITLE OF INVENTION: Genes Encoding No. US20020151709A1el Proteins With
; TITLE OF INVENTION: Pesticidal Activity Against Coleopterans
; FILE REFERENCE: 35718/237005
; CURRENT APPLICATION NUMBER: US/10/032,717
; CURRENT FILING DATE: 2001-10-23
; PRIOR APPLICATION NUMBER: 60/242,838
; PRIOR FILING DATE: 2000-10-24
; NUMBER OF SEQ ID NOS: 48
; SOFTWARE: FastSEQ for Windows Version 4.0
; SEQ ID NO 10
; LENGTH: 669
; TYPE: PRT
; ORGANISM: Artificial Sequence
; FEATURE:
; OTHER INFORMATION: Maize optimized Cry1218-1
US-10-032-717-10

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RESULT 4
US-10-032-717-16
; Sequence 16, Application US/10032717
; Patent No. US20020151709A1
; GENERAL INFORMATION:
; APPLICANT: Andre R. Abad
; APPLICANT: Nicholas B. Duck
; APPLICANT: Xiang Peng
; APPLICANT: Ronald D. Flannagan
; APPLICANT: Theodore W. Kahn
; APPLICANT: Lynn B. Sims
; TITLE OF INVENTION: Genes Encoding No. US20020151709A1el Proteins With
; TITLE OF INVENTION: Pesticidal Activity Against Coleopterans
; FILE REFERENCE: 35718/237005
; CURRENT APPLICATION NUMBER: US/10/032,717
; CURRENT FILING DATE: 2001-10-23
; PRIOR APPLICATION NUMBER: 60/242,838
; PRIOR FILING DATE: 2000-10-24
; NUMBER OF SEQ ID NOS: 48
; SOFTWARE: PastSEQ for Windows Version 4.0
; SEQ ID NO 16
; LENGTH: 669
; TYPE: PRT

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Scoring table: IDENTITY_NUC
Gapop 10.0 , Gapext 1.0

Searched: 441362 seqs, 153338381 residues

Total number of hits satisfying chosen parameters: 882724

Minimum DB seq length: 0
Maximum DB seq length: 2000000000

Post-processing: Minimum Match 0%
Maximum Match 100%
Listing first 45 summaries

Database : Issued_Patents_NA:*
1: /cgn2_6/ptodata/1/ina/5A_COMB.seq:*
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6: /cgn2_6/ptodata/1/ina/backfiles1.seq:*

Pred. No. is the number of results predicted by chance to have a score greater than or equal to the score of the result being printed, and is derived by analysis of the total score distribution.

SUMMARIES

Result No.	Score	% Query Match	Length	DB	ID	Description
1	2565.2	70.8	3507	1	US-08-315-468-3	Sequence 3, Appli
2	1812.2	50.0	3471	1	US-07-876-280-29	Sequence 29, Appl
3	1812.2	50.0	3471	1	US-07-812-180A-1	Sequence 1, Appli
4	1812.2	50.0	3471	1	US-08-315-468-1	Sequence 1, Appli
5	1812.2	50.0	3471	4	US-07-941-650A-1	Sequence 1, Appli
6	1180	32.6	3797	1	US-07-915-203-1	Sequence 1, Appli
7	1180	32.6	3797	1	US-08-272-887-1	Sequence 1, Appli
8	1180	32.6	3797	1	US-08-789-449-1	Sequence 1, Appli
9	883.4	24.4	4344	2	US-08-532-547-4	Sequence 4, Appli
10	883.4	24.4	4344	2	US-08-379-656B-4	Sequence 4, Appli
11	883.4	24.4	4344	3	US-08-455-838-4	Sequence 4, Appli
12	883.4	24.4	4344	3	US-09-019-809-4	Sequence 4, Appli
13	883.4	24.4	4344	4	US-09-471-177-4	Sequence 4, Appli
14	882.8	24.4	3471	4	US-09-002-285-73	Sequence 73, Appl
15	848.6	23.4	3759	1	US-08-542-921-1	Sequence 1, Appli
16	848.6	23.4	3759	2	US-08-880-685-1	Sequence 1, Appli
17	848.6	23.4	3759	2	US-08-880-684-1	Sequence 1, Appli
18	822.2	22.7	3453	4	US-09-002-285-75	Sequence 75, Appl
19	734.2	20.3	3411	4	US-09-002-285-77	Sequence 77, Appl
20	733.6	20.3	3414	1	US-07-973-320-3	Sequence 3, Appli
21	731.4	20.2	3414	1	US-07-973-320-1	Sequence 1, Appli
22	678	18.7	3934	1	US-08-100-709-3	Sequence 3, Appli
23	678	18.7	3934	1	US-08-176-865-3	Sequence 3, Appli
24	678	18.7	3934	1	US-08-474-038-3	Sequence 3, Appli
25	678	18.7	3934	2	US-08-779-046-3	Sequence 3, Appli
26	678	18.7	3934	2	US-08-881-340-3	Sequence 3, Appli
27	673.6	18.6	4074	1	US-08-377-690-1	Sequence 1, Appli
28	659.2	18.2	3684	1	US-08-448-170-7	Sequence 7, Appli
29	659.2	18.2	3684	3	US-08-961-803-5	Sequence 5, Appli
30	618.6	17.1	3567	6	5188960-5	Patent No. 5188960
31	615.4	17.0	3567	2	US-08-980-071-5	Sequence 5, Appli
32	615.4	17.0	3567	2	US-08-980-071-58	Sequence 58, Appl
33	615.4	17.0	3567	2	US-08-757-536-5	Sequence 5, Appli
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35	615.4	17.0	3567	3	US-09-314-093-58	Sequence 58, Appl
36	615.4	17.0	3567	3	US-09-250-848-5	Sequence 5, Appli
37	615.4	17.0	3567	4	US-09-251-885-5	Sequence 5, Appli
38	615.4	17.0	3567	4	US-09-337-635-5	Sequence 5, Appli
39	615.4	17.0	3567	4	US-09-337-635-58	Sequence 58, Appl
40	615.4	17.0	3567	4	US-09-337-280-5	Sequence 5, Appli
41	615.4	17.0	3567	4	US-09-337-280-58	Sequence 58, Appl
42	613.8	17.0	3567	1	US-08-602-737-1	Sequence 1, Appli
43	613.8	17.0	3567	2	US-08-980-071-1	Sequence 1, Appli
44	613.8	17.0	3567	2	US-08-980-071-9	Sequence 9, Appli
45	613.8	17.0	3567	2	US-08-980-071-11	Sequence 11, Appl

ALIGNMENTS

Instant SEQ ID NO:1 vs SEQ ID NO:3 of Michaels et al

RESULT 1
US-08-315-468-3
; Sequence 3, Application US/08315468
; Patent No. 5554534
; GENERAL INFORMATION:
; APPLICANT: Michaels, Tracy Ellis
; APPLICANT: Poncerrada, Luis
; APPLICANT: Narva, Kenneth E.
; TITLE OF INVENTION: Process for Controlling Scarab Pests
; TITLE OF INVENTION: with Bacillus thuringiensis Isolates
; NUMBER OF SEQUENCES: 6
; CORRESPONDENCE ADDRESS:
; ADDRESSEE: David R. Saliwanchik
; STREET: 2421 N.W. 41st Street, Suite A-1
; CITY: Gainesville
; STATE: FL
; COUNTRY: USA
; ZIP: 32606
; COMPUTER READABLE FORM:
; MEDIUM TYPE: Floppy disk
; COMPUTER: IBM PC compatible
; OPERATING SYSTEM: PC-DOS/MS-DOS
; SOFTWARE: PatentIn Release #1.0, Version #1.25
; CURRENT APPLICATION DATA:
; APPLICATION NUMBER: US/08/315,468
; FILING DATE:
; CLASSIFICATION: 424
; PRIOR APPLICATION DATA:
; APPLICATION NUMBER: US/08/014,941
; FILING DATE: 01 FEB 1993
; APPLICATION NUMBER: 07/828,430
; FILING DATE: 30-JAN-1992
; PRIOR APPLICATION DATA:
; APPLICATION NUMBER: 07/808,316
; FILING DATE: 16-DEC-1991
; ATTORNEY/AGENT INFORMATION:
; NAME: Saliwanchik, David R.
; REGISTRATION NUMBER: 31,794
; REFERENCE/DOCKET NUMBER: MA73.C2
; TELECOMMUNICATION INFORMATION:
; TELEPHONE: 904-375-8100
; TELEFAX: 904-372-5800
; INFORMATION FOR SEQ ID NO: 3:
; SEQUENCE CHARACTERISTICS:
; LENGTH: 3507 base pairs
; TYPE: nucleic acid
; STRANDEDNESS: double
; TOPOLOGY: linear
; MOLECULE TYPE: DNA (genomic)
; HYPOTHETICAL: NO

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;    ANTI-SENSE:  NO
;    ORIGINAL SOURCE:
;      ORGANISM:  Bacillus thuringiensis
;      STRAIN:  kumamotoensis
;      INDIVIDUAL ISOLATE:  50C
;    IMMEDIATE SOURCE:
;      LIBRARY:  LambdaGEM-11(tm) library of L. Foncerrada
;      CLONE:  50C(b)
US-08-315-468-3

  Query Match             70.8%;  Score 2565.2;  DB 1;  Length 3507;
  Best Local Similarity   85.1%;  Pred. No. 0;
  Matches 2906;  Conservative    0;  Mismatches  493;  Indels   15;  Gaps    3;

Qy       1 ATGAGTCCAAATAATCAAAATGAATATGAAATTATAGATGCGACACCTTCTACTTCTGTA 60
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Qy     121 GATTATAAAGATTATTTAAAAATGTCTGCGGGAAATGCTAGTGAATACCCTGGTTCACCT 180
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Qy     181 GAAGTACTTGTTAGCGGACAAGATGCAGCTAAGGCCGCAATTGATATAGTAGGTAAATTA 240
Db     181 GAGGTATTTCTAAGCGAGCAAGATGCAGTTAAGGCCGCAATTGATATAGTAGGTAAATTA 240

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Db     361 GAACTCATTAATCAAAAAATAGCAGAATATGCAAGGAATAAAGCGCTTTCGGAATTGGAA 420

Qy     421 GGATTAGGTAATAATTACCAATTATATCTAACTGCGCTTGAAGAATGGAAGAAAATCCA 480
Db     421 GGGCTAGGGAATAATTACCAATTATATCTAACTGCGCTTGAAGAGTGGAAAGAAAATCCA 480

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Qy     541 ACGCAATATATGCCATCTTTTAGAGTGACAAATTTTGAAGTACCATTCCTTACTGTATAT 600
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Db     601 ACAATGGCAGCAAACCTACATTTACTTTTATTAAGGGACGCATCAATTTTTGGAGAAGAA 660

Qy     661 TGGGGATGGTCAACAACTACTATTAATAACTATTATGATCGTCAAATGAAACTTACTGCA 720
Db     661 TGGGGATTGTCTACAAGCACTATTAATAACTACTATAATCGTCAAATGAAACTTACTGCA 720

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Db     721 GAATATTCTGACCACTGTGTAAAGTGGTATGAAACTGGTTTAGCAAAATTAAAAGGCTCG 780

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Db     781 AGCGCTAAACAATGGATTGACTATAACCAATTCCGTAGAGAAATGACATTGACGGTGTTA 840

Qy     841 GATGTTGTTGCATTATTCCCAAATTATGACACACGCACGTACCCAATGGAAACGAAAGCA 900
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Qy     901 CAACTAACAAGGGAAGTATATACAGATCCACTGGGCGCGGTAAACGTGTCTTCAATTGGT 960
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Qy     961 TCCTGGTATGACAAAGCACCTTCTTTCGGAGTGATAGAATCATCCGTTATTCGACCACCC 1020
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Qy    1021 CATGTATTTGATTATATAACGGGACTCACAGTGTATACACAATCAAGAAGCATTTCTTCC 1080
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Qy    1141 AGTAATCTTCAACAAATGTATGGAACTAATCAAAATCTACACAGCACTAGTACCTTTGAT 1200
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Db    1381 CGGACAAGAGATTCGGAATTAGAATTGCCTCCAGAAACTTCAGGTCAACCAAATTACGAG 1440

Qy    1441 TCATATAGCCATAGATTATGTCATATCACAAGTATTCCCGCGACGGGTAACACTACCGGA 1500
Db    1441 TCATATAGCCATAGATTAGGTCATATTACATTTATTTACTCCA---GTTCAACTAGCACG 1497

Qy    1501 TTAGTACCTGTATTTTCTTGGACACATCGAAGTGCAGATTTAAACAATACAATATATTCA 1560
Db    1498 TATGTACCTGTATTTTCTTGGACACATCGGAGTGCAGATCTAACAAATACAGTTAAAAGT 1557

Qy    1561 GATAAAATCACTCAAATTCCGGCCGTTAAATGTTGGGATAATTTACCGTTTGTTCCAGTG 1620
Db    1558 GGCGAAATCACCCAAATACCAGGGGGCAAGTCTAGCACCATAGGCAGAAATACTTATATA 1617

Qy    1621 GTAAAAGGACCAGGACATACAGGAGGGGATTTATTACAGTATAATAGAAGTACTGGTTCT 1680
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Db    1678 TGTGAGTTTCAGATGATCTTTCCAGAGTCTCAACGATTCCGTATTCGGATTCGTTACGCT 1737

Qy    1741 GTAAGACTGAGATATGCTACTGATGCAGATATTGTATTGCATGTAAACGATGCTCAGATT 1800
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Qy    1801 CAGATGCCAAAAACAATGAACCCAGGTGAGGATCTGACATCTAAAACTTTTAAAGTTGCA 1860
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Qy    1861 GATGCTATCACAACATTAAATTTAGCAACAGATAGTTCGCTAGCATTGAAACATAATTTA 1920
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Qy    1921 GGTGAAGACCCTAATTCAACATTATCTGGTATAGTTTACGTTGACCGAATCGAATTCATC 1980
Db    1915 GGTATACAAACGAATACAAATTTATTTATTTTA---------GACCGAATCGAATTCATC 1965
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Db   2026 GCCTTGTTTACGAATACAAAAGATGGATTACAGCCAGGTGTAACGGATTATGAAGTAAAT 2085

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Db   2086 CAAGCGGCCAACTTAGTGGAATGCCTATCGGATGATTTGTATCCAAATGAAAAACGATTG 2145

Qy   2161 TTATTTGATGCAGTGAGAGAGGCAAAACGCCTCAGTGAGGCACGTAATTTGCTTCAAGAT 2220
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Db   2146 TTATTTGATGCAGTGAGAGAGGCAAAACGACTTAGCGAGGCACGGAACTTACTACAAGAT 2205

Qy   2221 CCAGATTTCCAAGAGATAAATGGAGAAAATGGCTGGACGGCAAGTACGGGAATTGAGGTT 2280
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Qy   2281 ATAGAAGGGGATGCTTTATTCAAAGGGCGTTATCTACGCCTACCAGGTGCGAGAGAAATA 2340
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Qy   2341 GATACGGAAACGTATCCAACGTATCTGTATCAAAAAGTAGAGGAAGGTGTATTAAAACCA 2400
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Qy   2521 TCTCCTGTTAACTCGGATGGTAGTATCAATCGATGCAGCGAACAAAAGTATGTGAATAGC 2580
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Qy   2581 CGTTTAGAAGTAGAAAACCGTTCTGGTGAAGCGCATGAGTTCTCTATTCCTATTGATACA 2640
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Db   2686 GAGGGATACGCAACACTTGGAAATCTTGAATTGGTCGAAGAGGGACCTTTGTCAGGAGAC 2745

Qy   2761 GCATTAGAACGCTTGCAAAGAGAAGAACAACAGTGGAAGATTCAAATGACAAGAAGACGT 2820
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Qy   2821 GAAGAAACAGATAGAAGGTATATGGCATCGAAACAAGCGGTAGATCGTTTATATGCCGAT 2880
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Qy   2941 CTGATACAGTCCATTCCTTACGTATATAACGAAATGTTCCCAGAAATACCAGGGATGAAC 3000
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Qy   3001 TATACGAAGTTTACAGAATTAACAGATCGACTCCAACAAGCGTGGAGTTTGTATGATCAG 3060
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Qy   3061 CGAAATGCCATACCAAATGGTGATTTTCGAAATGGGTTAAGTAATTGGAATGCAACGCCT 3120
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Db   3046 CGAAACGCTATACCAAATGGAGATTACCGAAATGAATTAAGTAATTGGAATACAACATCT 3105

Qy   3121 GGCGTAGAAGTACAACAAATCAATCATACATCTGTCCTTGTGATTCCAAACTGGGATGAG 3180
          || ||  | ||||||||||||||||||||||||||||||||||||||||||||||| ||||
Db   3106 GGTGTGAATGTACAACAAATCAATCATACATCTGTCCTTGTGATTCCAAACTGGAATGAA 3165

Qy   3181 CAAGTTTCGCAACAGTTTACAGTTCAACCGAATCAAAGATATGTGTTACGAGTTACTGCG 3240
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Qy   3241 AGAAAAGAAGGGGTAGGAAATGGATATGTAAGTATCCGTGATGGTGGAAATCAAACAGAA 3300
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Db   3226 AGAAAAGAAGGGGTAGGAAATGGATATGTAAGTATTCGTGATGGTGGAAATCAATCAGAA 3285

Qy   3301 ACGCTTACTTTTAGTGCAAGCGATTATGATACAAATGGAATGTATAATACGCAAGTGTCC 3360
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Db   3346 AATACAAACGGATATAACACAAATAGTGTGTACATGATCAAACCGGCTATATCA 3399


RESULT 2
US-07-876-280-29
; Sequence 29, Application US/07876280
; Patent No. 5262158
;  GENERAL INFORMATION:
;    APPLICANT:  Payne, Jewel M.
;    APPLICANT:  Cannon, Raymond J.C.
;    APPLICANT:  Bagley, Angela L.
;    TITLE OF INVENTION:  No. 5262158el Bacillus thuringiensis Isolates for
;    TITLE OF INVENTION:  Controlling Acarides
;    NUMBER OF SEQUENCES:  30
;    CORRESPONDENCE ADDRESS:
;      ADDRESSEE:  David R. Saliwanchik
;      STREET:  2421 N.W. 41st Street, Suite A-1
;      CITY:  Gainesville
;      STATE:  FL
;      COUNTRY:  USA
;      ZIP:  32606
;    COMPUTER READABLE FORM:
;      MEDIUM TYPE:  Floppy disk
;      COMPUTER:  IBM PC compatible
;      OPERATING SYSTEM:  PC-DOS/MS-DOS
;      SOFTWARE:  PatentIn Release #1.0, Version #1.25
;    CURRENT APPLICATION DATA:
;      APPLICATION NUMBER:  US/07/876,280
;      FILING DATE:  19920430
;      CLASSIFICATION:  514
;    ATTORNEY/AGENT INFORMATION:
;      NAME:  Saliwanchik, David R.
;      REGISTRATION NUMBER:  31,794
;      REFERENCE/DOCKET NUMBER:  M/S 104
;    TELECOMMUNICATION INFORMATION:
;      TELEPHONE:  904-375-8100
;      TELEFAX:  904-372-5800
;  INFORMATION FOR SEQ ID NO:  29:
;    SEQUENCE CHARACTERISTICS:
;      LENGTH:  3471 base pairs
;      TYPE:  NUCLEIC ACID
;      STRANDEDNESS:  double
;      TOPOLOGY:  linear
;    MOLECULE TYPE:  DNA (genomic)
;    HYPOTHETICAL:  NO
;    ANTI-SENSE:  NO
;    ORIGINAL SOURCE:
;      ORGANISM:  Bacillus thuringiensis
;      STRAIN:  kumamotoensis
;      INDIVIDUAL ISOLATE:  PS50C
;    IMMEDIATE SOURCE:
;      CLONE:  E. coli NM522(pMYC2320) NRRL B-18769
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GenCore version 5.1.3

OM protein - protein search, using sw model

Run on: January 7, 2003, 05:12:43 ; Search time 26 Seconds
(without alignments)
1364.771 Million cell updates/sec

Title: US-10-032-717-2
Perfect score: 6332
Sequence: 1 MSPNNQNEYEIIDATPSTSV.....MSETEGTFYIESVELIVDVE 1206

Scoring table: BLOSUM62
Gapop 10.0 , Gapext 0.5

Searched: 262574 seqs, 29422922 residues

Total number of hits satisfying chosen parameters: 262574

Minimum DB seq length: 0
Maximum DB seq length: 2000000000

Post-processing: Minimum Match 0%
Maximum Match 100%
Listing first 45 summaries

Database : Issued_Patents_AA:*
1: /cgn2_6/ptodata/1/iaa/5A_COMB.pep:*
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Pred. No. is the number of results predicted by chance to have a score greater than or equal to the score of the result being printed, and is derived by analysis of the total score distribution.

SUMMARIES

Result No.	Score	% Query Match	Length	DB	ID	Description
1	4959.5	78.3	1169	1	US-08-315-468-4	Sequence 4, Appli
2	3950.5	62.4	1157	1	US-07-876-280-30	Sequence 30, Appl
3	3950.5	62.4	1157	1	US-07-812-180A-2	Sequence 2, Appli
4	3950.5	62.4	1157	1	US-08-315-468-2	Sequence 2, Appli
5	3950.5	62.4	1157	4	US-07-941-650A-2	Sequence 2, Appli
6	3021	47.7	1149	1	US-07-915-203-2	Sequence 2, Appli
7	3021	47.7	1149	1	US-08-272-887-2	Sequence 2, Appli
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27	2353.5	37.2	1227	1	US-08-448-170-8	Sequence 8, Appli
28	2353.5	37.2	1227	4	US-08-961-803-9	Sequence 9, Appli
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ALIGNMENTS

Instant SEQ ID NO: 1 vs protein encoded by SEQ ID NO: 3 of Michaels et al

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; Sequence 4, Application US/08315468
; Patent No. 5554534
; GENERAL INFORMATION:
;   APPLICANT: Michaels, Tracy Ellis
;   APPLICANT: Foncerrada, Luis
;   APPLICANT: Narva, Kenneth E.
;   TITLE OF INVENTION: Process for Controlling Scarab Pests
;   TITLE OF INVENTION: with Bacillus thuringiensis Isolates
;   NUMBER OF SEQUENCES: 6
;   CORRESPONDENCE ADDRESS:
;     ADDRESSEE: David R. Saliwanchik
;     STREET: 2421 N.W. 41st Street, Suite A-1
;     CITY: Gainesville
;     STATE: FL
;     COUNTRY: USA
;     ZIP: 32606
;   COMPUTER READABLE FORM:
;     MEDIUM TYPE: Floppy disk
;     COMPUTER: IBM PC compatible
;     OPERATING SYSTEM: PC-DOS/MS-DOS
;     SOFTWARE: PatentIn Release #1.0, Version #1.25
;   CURRENT APPLICATION DATA:
;     APPLICATION NUMBER: US/08/315,468
;     FILING DATE:
;     CLASSIFICATION: 424
;   PRIOR APPLICATION DATA:
;     APPLICATION NUMBER: US/08/014,941
;     FILING DATE: 01 FEB 1993
;     APPLICATION NUMBER: 07/828,430
;     FILING DATE: 30-JAN-1992
;   PRIOR APPLICATION DATA:
;     APPLICATION NUMBER: 07/808,316
;     FILING DATE: 16-DEC-1991
;   ATTORNEY/AGENT INFORMATION:
;     NAME: Saliwanchik, David R.
;     REGISTRATION NUMBER: 31,794
;     REFERENCE/DOCKET NUMBER: MA73.C2
;   TELECOMMUNICATION INFORMATION:
;     TELEPHONE: 904-375-8100
;     TELEFAX: 904-372-5800
; INFORMATION FOR SEQ ID NO: 4:
;   SEQUENCE CHARACTERISTICS:
;     LENGTH: 1169 amino acids
;     TYPE: amino acid
;     STRANDEDNESS: single
;     TOPOLOGY: linear
;   MOLECULE TYPE: protein
;   HYPOTHETICAL: YES
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;    ANTI-SENSE:  NO
;    ORIGINAL SOURCE:
;      ORGANISM:  Bacillus thuringiensis
;      STRAIN:  kumamotoensis
;      INDIVIDUAL ISOLATE:  50C
;    IMMEDIATE SOURCE:
;      LIBRARY:  LambdaGEM-11 library of L. Foncerrada
;      CLONE:  50C(b)
US-08-315-468-4

  Query Match             78.3%;  Score 4959.5;  DB 1;  Length 1169;
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  Matches  967;  Conservative   68;  Mismatches  128;  Indels   49;  Gaps    8;

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RESULT 2
US-07-876-280-30
; Sequence 30, Application US/07876280
; Patent No. 5262158
;  GENERAL INFORMATION:
;    APPLICANT:  Payne, Jewel M.
;    APPLICANT:  Cannon, Raymond J.C.
;    APPLICANT:  Bagley, Angela L.
;    TITLE OF INVENTION:  No. 5262158el Bacillus thuringiensis Isolates for
;    TITLE OF INVENTION:  Controlling Acarides
;    NUMBER OF SEQUENCES:  30
;    CORRESPONDENCE ADDRESS:
;      ADDRESSEE:  David R. Saliwanchik
;      STREET:  2421 N.W. 41st Street, Suite A-1
;      CITY:  Gainesville
;      STATE:  FL
;      COUNTRY:  USA
;      ZIP:  32606
;    COMPUTER READABLE FORM:
;      MEDIUM TYPE:  Floppy disk
;      COMPUTER:  IBM PC compatible
;      OPERATING SYSTEM:  PC-DOS/MS-DOS
;      SOFTWARE:  PatentIn Release #1.0, Version #1.25
;    CURRENT APPLICATION DATA:
;      APPLICATION NUMBER:  US/07/876,280
;      FILING DATE:  19920430
;      CLASSIFICATION:  514
;    ATTORNEY/AGENT INFORMATION:
;      NAME:  Saliwanchik, David R.
;      REGISTRATION NUMBER:  31,794
;      REFERENCE/DOCKET NUMBER:  M/S 104
;    TELECOMMUNICATION INFORMATION:
;      TELEPHONE:  904-375-8100
;      TELEFAX:  904-372-5800
;  INFORMATION FOR SEQ ID NO:  30:
;    SEQUENCE CHARACTERISTICS:
;      LENGTH:  1157 amino acids
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;      STRANDEDNESS:  single
;      TOPOLOGY:  linear
;    MOLECULE TYPE:  protein
;    HYPOTHETICAL:  YES
;    ANTI-SENSE:  NO
;    ORIGINAL SOURCE:
;      ORGANISM:  Bacillus thuringiensis
;      STRAIN:  kumamotoensis
;      INDIVIDUAL ISOLATE:  PS50C
```

Appendix B

Journal of Applied Microbiology 2003, 95, 23–28

Cloning and study of the expression of a novel *cry11a*-type gene from *Bacillus thuringiensis* subsp. *kurstaki*

S. Tounsi, N. Zouari and S. Jaoua

Laboratoire des Biopesticides, Centre de Biotechnologie de Sfax, Sfax, Tunisia

2002/239: received 6 June 2002, revised 13 February 2003 and accepted 21 February 2003

ABSTRACT

S. TOUNSI, N. ZOUARI AND S. JAOUA. 2003.

Aims: Cloning and expression of a new *cry11a*-type gene of *Bacillus thuringiensis*.

Methods and Results: PCR amplification, using gene *cry11*-specific primers revealed the presence of such a gene in the strain BNS3 of *Bacillus thuringiensis* subsp. *kurstaki*. The cloning and sequencing from BNS3 of the *cry11a*-type gene, called *crybns3-3*, showed an open reading frame of 2160-bp, encoding a protein of 719 amino acid residues. Both nucleotide and amino acid sequences similarity analysis revealed that the *crybns3-3* is a new *cry11a*-type gene, presenting several differences from the *cry11a*-type genes. The study of the expression of *crybns3-3* by Northern blot and RT-PCR showed that it was transcribed. The expression of *crybns3-3* under the control of BtI and BtII promoters revealed that Crybns3-3 would co-crystallize with the endogenous delta-endotoxins.

Conclusions: *crybns3-3* is a novel *cry11a* gene isolated from *B. thuringiensis* subsp. *kurstaki* strain BNS3.

Significance and Impact of the Study: The characteristics of *crybns3-3* indicate that it is a new *cry11a*-type gene. Amino acid residue substitutions presented in Crybns3-3 could be exploited for both toxicity and specificity studies. Crybns3-3 would interact and co-crystallize at least partially with the endogenous delta-endotoxins of BNS3, and then participate in the formation of the parasporal crystal inclusions.

Keywords: *Bacillus thuringiensis*, cloning, *cry11a*, DNA sequence, expression.

INTRODUCTION

Bacillus thuringiensis (Bt) is a Gram-positive bacterium characterized by the production of insecticidal crystal proteins during sporulation, exhibiting a wide variety of insecticidal specificities towards lepidopteran, coleopteran and dipteran insect species (Höfte and Whiteley 1989). These proteins are termed delta-endotoxins because of their intracellular location and have been used for many years as successful biological insecticides. Delta-endotoxins encoded by *cry* genes have been classified as Cry1-2-3... proteins, depending on their host specificity and their amino acid sequences (Höfte and Whiteley 1989). The occurrence of insect resistance to Cry proteins (Tabashnik *et al.* 1990) has been attributed to a reduction in the affinity of the proteolytically activated Cry toxin for binding to the membrane of mid-gut epithelial cells of the larvae (Tabashnik *et al.* 1990). In order to prevent the continuous development of resistance to Bt insecticides, a search for novel Bt strains (Zouari *et al.* 2002a) harbouring new *cry* genes has been undertaken (Shin *et al.* 1995). Thus, Tailor *et al.* (1992) reported a new class of *cry* genes encoding proteins that could not be found in solubilized Bt crystal preparations. Such a gene is designated *cry11a* (Crickmore *et al.* 1998) on the basis of the following criteria: (i) it has 62% sequence homology with *cry1Ba* (Tailor *et al.* 1992) and (ii) when expressed in *Escherichia coli*, the encoded 81 kDa protein, showed specificity and insecticidal activity against lepidopterans (*Ostrinia nubilalis*) and coleopterans (*Leptinotarsa decimlineata*) (Tailor *et al.* 1992). However, other groups (Shin *et al.* 1995) have reported the cloning and characterization of silent *cry11a*-type genes in different Bt strains. Recently, Masson *et al.* (1998), reported that the Bt subsp. *aizawai* strain HD133 expressed a *cry11a*-type gene at the early stages of the sporulation phase.

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In this paper, we report the cloning, the nucleotide sequence and the study of the expression of a new *cry11a*-type gene named *crybns3-3*, isolated from the strain BNS3 (Tounsi *et al.* 1999) of Bt, and whose promoter has been previously identified (Tounsi and Jaoua 2002).

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The strain BNS3 employed in the present work is Bt *kurstaki* subsp. H3a, 3b, 3c (Tounsi *et al.* 1999). This strain produces bipyramidal parasporal crystals exhibiting insecticidal activities (Tounsi *et al.* 1999). This strain was selected for the large-scale production of bioinsecticides (Zouari *et al.* 1998, 2002b). The strain BNS3Cry$^-$ is an acrystalliferous strain derivative of BNS3 (Tounsi *et al.* 1999). *Escherichia coli* DH5α obtained from Amersham (Sunnyvale, CA, USA), was used as a cloning host. Plasmid pBluescript II KS(+) (Stratagene, La Jolla, CA, USA) was the vector used to construct the genomic library. Plasmid pHTBlue, a derivative of pHT3101, was made in the laboratory by substituting the multiple cloning site of the latter for that of the plasmid pBluescript II KS (unpublished data). It was used to study the expression of *cry* genes in Bt. Plasmid pHTB*crybns3-3* was constructed in this work by cloning the gene *crybns3-3* of BNS3 in the plasmid pHTBlue, downstream of the BtI and BtII promoters. Strains BNS3(pHTB*crybns3-3*) and BNS3Cry$^-$(pHTB*crybns3-3*) were constructed in this work by transferring the plasmid pHTB*crybns3-3*, respectively, to BNS3 and BNS3Cry$^-$. LB broth (Sambrook *et al.* 1989) was used for growth of Bt and *E. coli*. Glucose medium was used for delta-endotoxin production by Bt strains (Zouari *et al.* 1998).

Preparation of DNA and DNA probe, and PCR amplification

Plasmid DNA was extracted from Bt strains using the alkali lysis method including a lysozyme treatment step (Sambrook *et al.* 1989). Primers cry5A and cry5B (Shin *et al.* 1995) were synthesized by the Centre de Génétique Moléculaire, CNRS, GENSET, Orsay, France. PCR was accomplished as described by Jaoua *et al.* (1996). The *cry11a*-specific probe was prepared from BNS3, by PCR using primers cry5A and cry5B.

Nucleic acid hybridization

Restricted DNAs were size separated by electrophoresis in horizontal 0·8% agarose slab gels and transferred to nylon membrane (Hybond N$^+$; Amersham) (Sambrook *et al.* 1989). Prehybridization and hybridization of filters were carried out as described in Sambrook *et al.* (1989).

Cloning experiment, transformation procedures and DNA sequencing

Plasmid DNA purified from the BNS3 was digested with *Cla*I and *BamHI* and separated by electrophoresis through a 0·8% agarose gel. DNA fragments (3-kb) were ligated to pBluescript II KS digested with both *Cla*I and *BamH*I. Transformation of *E. coli* DH5α was performed according to the manufacturer's protocol (Bethesda Research Laboratories, Bethesda, MD, USA). Transformation of BNS3Cry$^-$ and BNS3 was accomplished by electroporation using a Bio-Rad Gene Pulser (BioRad, Hercules, CA, USA), according to Lereclus *et al.* (1989).

DNA sequences were obtained according to the dideoxy-chain termination method using the Thermo Sequenase cycle sequencing kit (Amersham). Sequence comparisons were performed using Blast through the NCBI E-mail server.

The DNA sequence of *crybns3-3* had been deposited in the EMBL and Gene Bank data bases under the accession number AJ315121.

RNA isolation, RT-PCR, Northern blot analysis

Total RNA from BNS3 and BNS3Cry$^-$ was isolated from cells grown in 10 ml of LB medium at 30°C for 15 h (Tounsi and Jaoua 2002). Bacterial cells were centrifuged at 5000 *g* for 5 min at 4°C. Pellets were suspended in 2 ml of buffer (25% w/v, sucrose; 50 mmol l^{-1} Tris–HCl (pH 8·0); 0·25 mmol l^{-1} EDTA) containing 1 mg ml^{-1} lysosyme, and incubated for 15 min at 37°C. The obtained proptoplasts were harvested by centrifugation and suspended in 0·6 ml lysis buffer (3 mmol l^{-1} EDTA; 200 mmol l^{-1} NaCl; 0·5% SDS) and 0·6 ml of phenol and centrifuged at 12 000 *g* for 5 min at 4°C. The aqueous phase containing total RNA was treated twice with phenol/chloroform/isoamylalcohol (25 : 24 : 1, by vol.). After precipitation with 100% ethanol, RNA pellets were washed with 1 ml 70% (v/v) ethanol, dried and dissolved in 50 μl of sterilized bi-distilled water treated with diethyl pyrocarbonate. RT-PCR experiments were accomplished by using ready-to-go RT-PCR kit (Amersham) and purified RNA was treated with DNase I (RNase-free, Amersham). For Northern blot analysis, 20 μg of total RNA was electrophoresed in 1% agarose gel containing 1·2 mol l^{-1} formaldehyde. The RNA was transferred to nylon membrane (Hybond N$^+$; Amersham) using 20× SSC (1× SSC is 0·15 mol l^{-1} NaCl, 0·015 mol l^{-1} sodium citrate).

Delta-endotoxin determination

Delta-endotoxin was determined according to Zouari *et al.* (1998).

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ATGAAACTAAAGAATCAAGATAAGCATCAAAGTTTTTCTAGCAATGCGAAAGTAGATAAAATCTCTACGGATTCACTAAAAAATGAAACAGATATAGAAT  100
 M  K  L  K  N  Q  D  K  H  Q  S  F  S  S  N  A  K  V  D  K  I  S  T  D  S  L  K  N  E  T  D  I  E
TACAAAACATTAATCATGAAGATTGTTTGAAAATGTCTGAGTATGAAAATGTAGAGCCGTTTGTTAGTGCATCAACAATTCAAACAGGTATTGGTATTGC  200
 L  Q  N  I  N  H  E  D  C  L  K  M  S  E  Y  E  N  V  E  P  F  V  S  A  S  T  I  Q  T  G  I  G  I  A
GGGTAAAATACTTGGTACCCTAGGCGTTCCTTTTGCAGGACAAGTAGCTAGTCTTTATAGTTTTATCTTAGGTGAGCTATGGCCTAAGGGGAAAAATCAA  300
  G  K  I  L  G  T  L  G  V  P  F  A  G  Q  V  A  S  L  Y  S  F  I  L  G  E  L  W  P  K  G  K  N  Q
TGGGAAATCTTTATGGAACATGTAGAAGAGATTATTAATCAAAAAATATCAACTTATGCAAGAAATAAAGCACTTACAGACTTGAAAGGATTAGGAGATG  400
 W  E  I  F  M  E  H  V  E  E  I  I  N  Q  K  I  S  T  Y  A  R  N  K  A  L  T  D  L  K  G  L  G  D
CCTTAGCTGTCTACCATGATTCGCTTGAAAGTTGGGTTGGAAATCGTAATAACACAAGGGCTAGGAGTGTTGTCAAGAGCCAATATATCGCATTAGAATT  500
A  L  A  V  Y  H  D  S  L  E  S  W  V  G  N  R  N  N  T  R  A  R  S  V  V  K  S  Q  Y  I  A  L  E  L
GATGTTCGTTCAGAAACTACCTTCTTTTGCAGTGTCTGGAGAGGAGGTACCATTATTACCGATATATGCCCAAGCTGCAAATTTACATTTGTTGCTATTA  600
  M  F  V  Q  K  L  P  S  F  A  V  S  G  E  E  V  P  L  L  P  I  Y  A  Q  A  A  N  L  H  L  L  L  L
AGAGATGCATCTATTTTTGGAAAAGAGTGGGGATTATCATCTTCAGAAATTTCAACATTTTATAACCGTCAAGTCGAACGAGCAGGAGATTATTCCGACC  700
 R  D  A  S  I  F  G  K  E  W  G  L  S  S  S  E  I  S  T  F  Y  N  R  Q  V  E  R  A  G  D  Y  S  D
ATTGTGTGAAATGGTATAGCACAGGTCTAAATAACTTGAGGGGTACAAATGCCGAAAGTTGGGTACGATATAATCAATTCCGTAGAGACATGACTTTAAT  800
H  C  V  K  W  Y  S  T  G  L  N  N  L  R  G  T  N  A  E  S  W  V  R  Y  N  Q  F  R  R  D  M  T  L  M
GGTACTAGATTTAGTGGCACTATTTCCAAGCTATGATACACAAATGTATCCAATTAAAACTACAGCCCAACTTACAAGAGAAGTATATACAGACGCAATT  900
  V  L  D  L  V  A  L  F  P  S  Y  D  T  Q  M  Y  P  I  K  T  T  A  Q  L  T  R  E  V  Y  T  D  A  I
GGGACAGTACATCCGCATCCAAGTTTTACAAGTACGACTTGGTATAATAATAATGCACCTTCGTTCTCTGCCATAGAGGCTGCTGTTGTTCGAAACCCGC  1000
 G  T  V  H  P  H  P  S  F  T  S  T  T  W  Y  N  N  N  A  P  S  F  S  A  I  E  A  A  V  V  R  N  P
ATCTACTCGATTTTCTAGAACAAGTTACAATTTACAGCTTATTAAGTCGATGGAGTAACACTCAGTATATGAATATGTGGGGAGGACATAAACTAGAATT  1100
H  L  L  D  F  L  E  Q  V  T  I  Y  S  L  L  S  R  W  S  N  T  Q  Y  M  N  M  W  G  G  H  K  L  E  F
CCGAACAATAGGAGGAACGTTAAATATCTCAACACAAGGATCTACTAATACTTCTATTAATCCTGTAACATTACCGTTCACTTCTCGAGACGTCTATAGG  1200
  R  T  I  G  G  T  L  N  I  S  T  Q  G  S  T  N  T  S  I  N  P  V  T  L  P  F  T  S  R  D  V  Y  R
ACTGAATCATTGGCAGGGCTGAATCTATTTTTAACTCAACCTGTTAATGGAGTACCTAGGGTTGATTTTCATTGGAAATTCGTCACACATCCGATCGCAT  1300
 T  E  S  L  A  G  L  N  L  F  L  T  Q  P  V  N  G  V  P  R  V  D  F  H  W  K  F  V  T  H  P  I  A
CTGATAATTTCTATTATCCAGGGTATGCTGGAATTGGGACGCAATTACAGGATTCAGAAAATGAATTACCACCTGAAGCAACAGGACAGCCAAATTATGA  1400
S  D  N  F  Y  Y  P  G  Y  A  G  I  G  T  Q  L  Q  D  S  E  N  E  L  P  P  E  A  T  G  Q  P  N  Y  E
ATCTTATAGTCATAGATTATCTCATATAGGACTCATTTCAGCATCACATGTGAAAGCATTGGTATATTCTTGGACGCATCGTAGTGCAGATCGTACAAAT  1500
  S  Y  S  H  R  L  S  H  I  G  L  I  S  A  S  H  V  K  A  L  V  Y  S  W  T  H  R  S  A  D  R  T  N
ACAATTGAGCCAAATAGCATTACACAAATACCATTAGTAAAAGCTTTCAATCTGTCTTCAGGTGCCGCTGTAGTGAGAGGACCAGGATTTACAGGTGGGG  1600
 T  I  E  P  N  S  I  T  Q  I  P  L  V  K  A  F  N  L  S  S  G  A  A  V  V  R  G  P  G  F  T  G  G
ATATCCTTCGAAGAACGAATACTGGTACATTTGGGGATATACGAGTAAATATTAATCCACCATTTGCACAAAGATATCGCGTGAGGATTCGCTATGCTTC  1700
D  I  L  R  R  T  N  T  G  T  F  G  D  I  R  V  N  I  N  P  P  F  A  Q  R  Y  R  V  R  I  R  Y  A  S
TACCACAGATTTACAATTCCATACGTCAATTAACGGTAAAGCTATTAATCAAGGTAATTTTTCAGCAACTATGAATAGAGGAGAGGACTTAGACTATAAA  1800
  T  T  D  L  Q  F  H  T  S  I  N  G  K  A  I  N  Q  G  N  F  S  A  T  M  N  R  G  E  D  L  D  Y  K
ACCTTTAGAACTGTAGGCTTTACCACTCCATTTAGCTTTTTAGATGTACAAAGTACATTCACAATAGGTGCTTGGAACTTCTCTTCAGGTAACGAAGTTT  1900
 T  F  R  T  V  G  F  T  T  P  F  S  F  L  D  V  Q  S  T  F  T  I  G  A  W  N  F  S  S  G  N  E  V
ATATAGATAGAATTGAATTTGTTCCGGTAGAAGTAACATATGAGGCAGAATATGATTTTGAAAAAGCGCAAGAGAAGGTTACTGCACTGTTTACATCTAC  2000
Y  I  D  R  I  E  F  V  P  V  E  V  T  Y  E  A  E  Y  D  F  E  K  A  Q  E  K  V  T  A  L  F  T  S  T
GAATCCAAGAGGATTAAAAACAGATGTAAAGGATTATCATATTGACCAGGTATCAAATTTAGTAGAGTCTCTATCAGATGAATTCTATCTTGATGAAAAG  2100
  N  P  R  G  L  K  T  D  V  K  D  Y  H  I  D  Q  V  S  N  L  V  E  S  L  S  D  E  F  Y  L  D  E  K
AGAGAATTATTCGAGATAGTTAAATACGCGAACGAACTCCATATTGAGCGTAACATGTAG                                        2160
R  E  L  F  E  I  V  K  Y  A  N  E  L  H  I  E  R  N  M  *
```

Fig. 1 Nucleotide sequence (EMBL accession number AJ315121) and deduced amino acid sequence of *arybns3-3*. The underlined ATG and TGA correspond, respectively, to the start and stop condons of *crybns3-3*. The predicted *Bacillus* secretion signal peptide has been italicized

RESULTS

Southern hybridization and cloning of a *cryIIa*-type gene from BNS3

The presence of a *cryIIa*-type gene in BNS3 was demonstrated by PCR amplification of an expected 0·7-kb fragment (Tounsi *et al.* 1999). Plasmid DNA of BNS3 digested with both *Cla*I and *BamH*I restriction enzymes, was analysed by Southern blot hybridization with the 0·7-kb radio-labelled probe. A band corresponding to 3-kb *Cla*I-*BamH*I restriction fragment was visualized on the autoradiogram (unpublished data). Thus, a library of the 3-kb *Cla*I-*BamH*I fragments was constructed from BNS3 plasmid DNA and screened at strong stringency conditions using the same 0·7-kb probe mentioned above. The restriction enzyme analysis of the positive recombinant clones revealed the presence of a *cryIIa*-type gene in BNS3. The cloned gene was called *crybns3-3*.

Sequence analysis of *crybns3-3*

Analysis of the nucleotide sequence (EMBL accession no. AJ315121) (Fig. 1) of the cloned 3-kb *Cla*I-*BamH*I fragment showed the presence of an open reading frame of 2160 bp encoding a protein of 719 amino acid residues having a predicted molecular mass of 81 kDa. The search for sequence similarity of *crybns3-3* with the known genes, using the Blast program, revealed several differences with the published *cryIIa*-type genes (Fig. 2). When compared with *cryIIa1* (Tailor *et al.* 1992), there were substitutions at positions 697 of T for G (transversion), 2133 of G for C (transversion) and 2134 of C for G (transversion), resulting in the substitution of Tyr^{233} for Asp (domain I), Lys^{711} for Asn and Gln^{712} for Glu (domain III). Compared with *cryIIa2* (Gleave *et al.* 1993), there were substitutions at positions 2133 of G for C and 2134 of C for G, resulting in the substitution of Lys^{711} for Asn and Gln^{712} for Glu (domain III). Compared with *cryIIa3* (Shin *et al.* 1995), there were substitutions at positions 697 of T for G, resulting in the substitution in the amino acids sequence of Tyr^{233} for Asp (domain I). Compared with *cryIIa4* (Kostichka *et al.* 1996), there were substitutions at positions 1328 of T for C (transition), 2133 of G for C, 2134 of C for G and 1398 of C for T, resulting in the substitution in the amino acids sequence of, respectively, Val^{443} for Ala (domain II), Lys^{711} for Asn and Gln^{712} for Glu (domain III).

Study of the expression of the *crybns3-3* gene in the wild strain BNS3

Although DNA dot-blot analysis and/or PCR analysis confirmed the presence of a *cryIIa*-type gene in most Bt strains, CryIIa proteins were not detected in solubilized

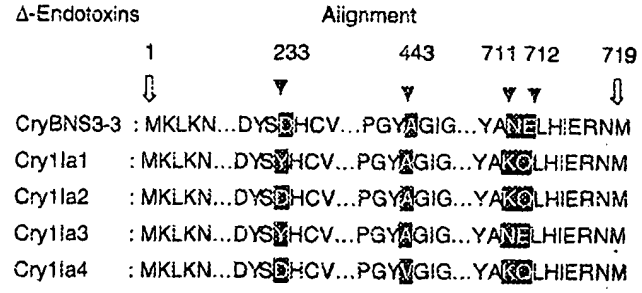

Fig. 2 Comparison of the amino acid sequence encoded by *crybns3-3* with those encoded by the other *cryIIa*-type genes. Only the regions containing differences are presented. The vertical downward arrows indicate amino acid position. The black boxes represent the residues showing variations

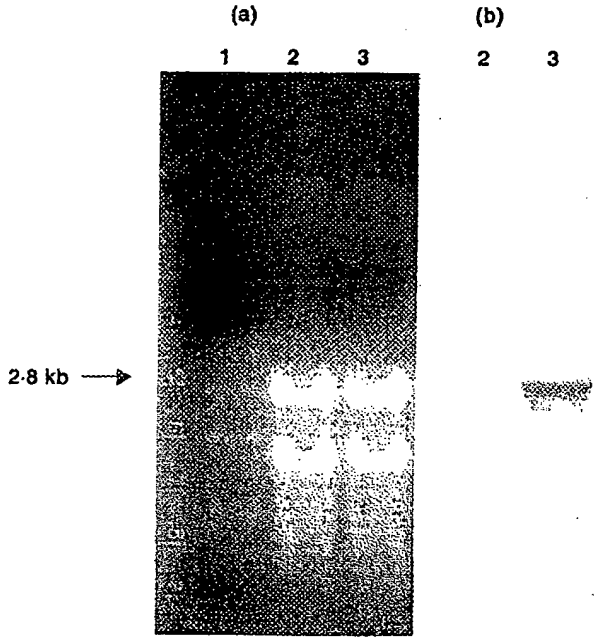

Fig. 3 Northern blot analysis of *crybns3-3* transcript (digitally processed). (a) Total RNA, extracted as described in material and methods from BNS3 and BNSCry$^-$, and electrophoresed in 1% agarose gel containing 1·2 mmol l^{-1} formaldehyde. Lanes: 1, RNA Molecular weight markers (Amersham): 9·46, 6·23, 3·91, 2·80, 1·90, 0·87, 0·56, 0·36-kb; 2, total RNA of BNS3Cry$^-$; 3, total RNA of BNS3. (b) Northern blot of RNAs hybridized to a *cryIIa*-specific probe (lane designation are the same as for panel a)

crystal preparations (Tailor *et al.* 1992; Gleave *et al.* 1993). However, Kostichka *et al.* (1996) and Masson *et al.* (1998) reported, respectively, that Bt AB88 expressed a *cryIIa*-type gene in early stationary phase and Bt HD133 expressed a *cryIIa*-type gene at both the T2 and T5 stages of the sporulation phase. In our case, Crybns3-3 proteins were not found by SDS-PAGE of crystal preparation (unpublished

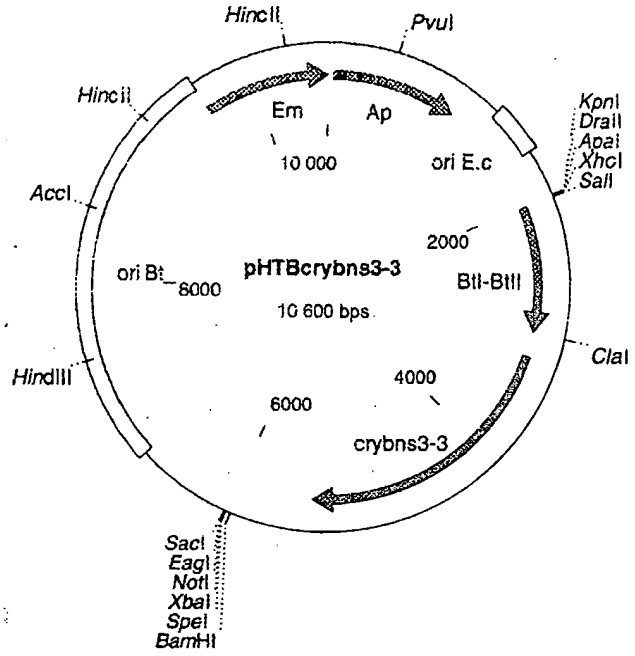

Fig. 4 Restriction map of the plasmid pHTB*crybns3-3*. The construction of the plasmid is described in the text. Open boxes contain the replication origins of, respectively, Bt (ori Bt) and *Escherichia coli* (ori E.c.). Filled arrows indicates the direction of transcription of the erythromycin resistance gene (Em), ampicillin resistance gene (Ap), *crybns3-3* gene and the orientation of the double promoters BtI and BtII

Table 1 Evidence of the Crybns3-3-mediated delta-endotoxin production improvement

Strain	CFU (10^7 C ml^{-1})	Production of delta-endotoxin (mg l^{-1})	Improvement of delta-endotoxin production
BNS3(pHTBlue)	100	960	Control
BNS3(PHTB*crybns3-3*)	100	1120	16·6 %

data). Analysis of total RNA of BNS3 by Northern blot demonstrated that BNS3 expressed a *cry11a*-type gene with a transcript size of 2·8-kb (Fig. 3). RT-PCR profile showed the amplification of an expected 0·7-kb fragment (using cry5A and cry5B primers), which confirm the expression of *crybns3-3* (unpublished data).

Expression of *crybns3-3* under the control of the BtI and BtII promoters

In order to improve the expression of *crybns3-3* during the early and later stages of sporulation, the *crybn3-3* was cloned downstream of the double promoters BtI and BtII (Sedlak *et al.* 2000). First, the 3-kb *ClaI-BamHI* fragment containing the *crybns3-3* gene was cloned in between the *Cla*I and *BamH*I sites of the plasmid pHTBlue. Then, a 1-kb *Sma*I fragment containing the BtI and BtII promoters obtained by PCR amplification-*Sma*I digestion (unpublished results) was cloned in the *Hinc*II site of the recombinant plasmid pHT*crybns3-3*. The resulting recombinant plasmid, containing the *crybns3-3* gene under the control of the BtI and BtII promoters designated pHTB*crybns3-3* (Fig. 4), was transferred by electroporation to both BNS3 and BNS3Cry$^-$. Microscopic examination of sporulated recombinant transformants BNS3Cry$^-$ (pHTB*crybns3-3*) revealed the lack of parasporal crystal inclusions. However, using polyclonal antibodies against *cry1A* genes, the Crybns3-3 was detected in the supernatant by ELISA. However, a comparison of delta-endotoxin production by the different strains (Table 1) showed that BNS3(pHTB*crybns3-3*) produced 16% more delta-endotoxins than BNS3(pHTBlue) used as a control.

DISCUSSION

It is known that Cry proteins, belonging to the same type, are closely related. In the present work we have isolated a novel *cry11a*-type gene, *crybns3-3*, encoding a protein of 719 amino acid residues, having a predicted molecular mass of 81 kDa. Hence, the Crybns3-3 protein sequence is different at several sites from those of the reported Cry11a proteins. As regards the effect of the reported substitutions in the *crybns3-3* gene, we could expect modifications in toxicity and specificity of the encoded delta-endotoxins. Deep investigations of such effects should be performed. Although delta-endotoxins encoded by *cry11a1* and *cry11a2* genes included single amino acid differences in domain I, they exhibited a different insecticidal activity spectrum, inferring different specificities (Gleave *et al.* 1993). Such results reinforce the idea that substitutions presented by *crybns3-3* could be of interest for studies on toxicity.

We demonstrated that BNS3 expressed a *cry11a*-type gene with a transcript size of 2·8-kb. By ELISA, the Crybns3-3 protein was detected at the early stages of sporulation, in the culture media of BNS3. This indicates that Crybns3-3 delta-endotoxin is secreted probably due to the presence of a putative signal peptide in the N-terminal domain I (Kostichka *et al.* 1996).

In order to improve its expression, *crybns3-3* was cloned downstream both of the promoters BtI and BtII in the shuttle vector pHTBlue, and transferred to both BNS3 and BNS3Cry$^-$. The lack of parasporal crystal inclusions in the sporulated recombinant transformants BNS3Cry$^-$ (pHTB*crybns3-3*) demonstrates that even when transcribed during the early and later stages of sporulation, the delta-endotoxin Crybns3-3 does not crystallize. The enhancement of delta-endotoxin production by BNS3(pHTB*crybns3-3*) clearly shows that Crybns3-3 does not crystallize when it is

alone, but can interact and co-crystallize partially with the endogenous delta-endotoxins of BNS3, and participate in the formation of parasporal crystal inclusions.

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